(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 April 2001 (26.04.2001)

PCT

(10) International Publication Number
WO 01/29073 A1

(51) International Patent Classification[7]: C07K 14/37, C12N 15/80, C12P 21/00, C12R 1/645

(21) International Application Number: PCT/US00/28903

(22) International Filing Date: 18 October 2000 (18.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/160,587 20 October 1999 (20.10.1999) US
09/487,558 19 January 2000 (19.01.2000) US

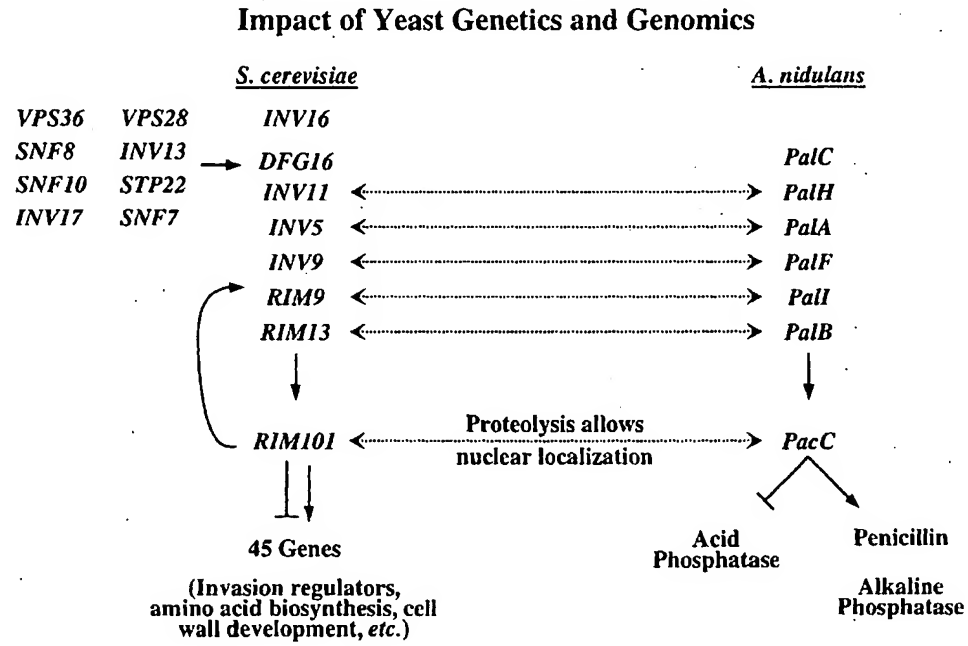
(71) Applicant: **MICROBIA, INC.** [US/US]; One Kendall Square, Building 1400W, Suite 1418, Cambridge, MA 01810 (US).

(72) Inventors: **BUSBY, Robert**; 84 Vine Street, Weymouth, MA 02188 (US). **DOTEN, Reed**; 1300 Worcester Road, Apt. P601, Framingham, MA 01702 (US). **CALI, Brian**; 100 Hillside Avenue, Arlington, MA 02476 (US). **HECHT, Peter**; 17 Duffield Road, Newton, MA 02466 (US). **HOLTZMAN, Doug**; 821 Centre Street, #6, Jamaica Plain, MA 02130 (US). **MADDEN, Kevin**; 40 Chestnut Street, Charlestown, MA 02129 (US). **MAXON, Mary**; 33 Grant Street, Somerville, MA 02145 (US). **MILNE, Todd**; 169 Mason Terrace, Brookline, MA 02446 (US). **NORMAN, Thea**; 26 Marion Road, Belmont, MA 02478 (US). **ROYER, John**; 16 Demar Road, Lexington, MA 02420 (US). **SALAMA, Sofie**; 696 Tremont Street, #2, Boston, MA 02118 (US). **SHERMAN, Amir**; 3 Joy Street, Apt. 10, Boston, MA 02108 (US). **SILVA, Jeff**; 95 Hale Street, Beverly, MA 01915 (US). **SUMMERS, Eric**; 204 Summit Avenue, Brookline, MA 02446 (US). **ZHANG, Lixin**; 15 Farmcrest Avenue, Lexington, MA 02421 (US). **MAYORGA, Maria**; 85 Elm Street, Belmont, MA 02478 (US). **FEIBELMAN, Toby**; 65 Allston Street, Cambridge, MA 20139 (US).

(74) Agents: **KEOWN, Wayne, A.** et al.; Hale and Dorr LLP, 60 State Street, Boston, MA 02109 (US).

[Continued on next page]

(54) Title: METHODS FOR IMPROVING SECONDARY METABOLITE PRODUCTION IN FUNGI



(57) Abstract: The invention relates to the production of secondary metabolites by fungi. More particularly, the invention relates to improvement of production of commercially important secondary metabolites by fungi. The invention provides methods for improving secondary metabolite production in a fungus, comprising modulating the expression of a gene involved in regulation of secondary metabolite production.

Best Available Copy

WO 01/29073 A1

(81) **Designated States** *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *With international search report.*

— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHODS FOR IMPROVING SECONDARY METABOLITE PRODUCTION IN FUNGI

BACKGROUND OF THE INVENTION

Field of the invention

The invention relates to the production of secondary metabolites by fungi. More particularly, the invention relates to improvement of production of commercially important secondary metabolites by fungi.

Summary of the related art

Secondary metabolite production by various fungi has been an extremely important source of a variety of therapeutically significant pharmaceuticals. B-lactam antibacterials such as penicillin and cephalosporin are produced by *Penicillium chrysogenum* and *Acremonium chrysogenum*, respectively, and these compounds are by far the most frequently used antibacterials (reviewed in Luengo and Penalva, Prog. Ind. Microbiol. 29: 603-38 (1994); Jensen and Demain, Biotechnology 28: 239-68 (1995); Brakhage, Microbiol. Mol. Biol. Rev. 62: 547-85 (1998)). Cyclosporin A, a member of a class of cyclic undecapeptides, is produced by *Tolypocladium inflatum*. Cyclosporin A dramatically reduces morbidity and increases survival rates in transplant patients (Borel, Prog. Allergy 38: 9-18 (1986)). In addition, several fungal secondary metabolites are cholesterol lowering drugs, including lovastatin that is made by *Aspergillus terreus* and several other fungi (Alberts *et al.*, Proc. Natl. Acad. Sci. USA 77: 3957-3961 (1980)). These and many other fungal secondary metabolites have contributed greatly to health care throughout the world (see Demain, Ciba Found Symp 171: 3-16 (1992); Bentley, Crit. Rev. Biotechnol. 19: 1-40 (1999)).

Unfortunately, many challenges are encountered between the detection of a secondary metabolite activity to production of significant quantities of pure drug. Thus, efforts have been made to improve the production of secondary metabolites by fungi. Some of these efforts have attempted to improve production by modification of the growth medium or the bioreactor used to carry out the fermentation. Buckland *et al.*, in Topics in Industrial Microbiology: Novel Microbial products for Medicine and Agriculture, pp. 161-169, Elsevier, Amsterdam (1989)

discloses improved lovastatin production by modification of carbon source and also teaches the superiority of a hydrofoil axial flow impeller in the bioreactor. Other efforts have involved strain improvements, either through re-isolation or random mutagenesis. Agathos *et al.*, J. Ind. Microbiol. 1: 39-48 (1986), teaches that strain improvement and process development together resulted in a ten-fold increase in cyclosporin A production. While important, studies of these types have still left much room for improvement in the production of secondary metabolites.

More recently, strains have been improved by manipulation of the genes encoding the biosynthetic enzymes that catalyze the reactions required for production of secondary metabolites. Penalva *et al.*, Trends Biotechnol. 16: 483-489 (1998) discloses that production strains of *P. chrysogenum* have increased copy number of the penicillin synthesis structural genes. Other studies have modulated expression of other biosynthetic enzyme-encoding genes, thereby affecting overall metabolism in the fungus. Mingo *et al.*, J. Biol. Chem. 21: 14545-14550 (1999), demonstrate that disruption of phacA, an enzyme in *A. nidulans* that catalyzes phenylacetate 2-hydroxylation, leads to increased penicillin production, probably by elimination of competition for the substrate phenylacetate. Similarly, disruption of the gene encoding aminoadipate reductase in *P. chrysogenum* increased penicillin production, presumably by eliminating competition for the substrate alpha-aminoadipate (Casquiero *et al.*, J. Bacteriol. 181: 1181-1188 (1999)).

Thus, genetic manipulation holds promise for improving production of secondary metabolites. Genetic manipulation to increase the activity of biosynthetic enzymes for secondary metabolite production or to decrease the activity of competing biosynthetic pathways has proven effective for improving production. Maximum benefit might be achieved by combining several strategies of manipulation. For example, modulating the expression of genes that regulate the biosynthetic enzyme-encoding genes might improve production. In addition, genetic manipulation could be used to impact upon the challenges that are encountered in the fermentor run or downstream processing (*e.g.* energy cost, specific production of desired metabolite, maximal recovery of metabolite, cost of processing waste from fermentations). There is, therefore, a need for methods for improving secondary metabolite production in a fungus, comprising modulating the expression of a gene involved in regulation of secondary metabolite production. Ideally, such methods should be able to provide increased yield, increased

productivity, increased efflux/excretion, decreased production of side products or non-desired secondary metabolites, altered strain characteristics and/or conditional lysis, or increased resistance to the deleterious effects of exposure to a secondary metabolite.

BRIEF SUMMARY OF THE INVENTION

The invention provides methods for improving secondary metabolite production in a fungus, comprising modulating the expression of a gene involved in regulation of secondary metabolite production. The methods according to the invention provide increased yield, increased productivity, increased efflux/excretion, decreased production of side products or non-desired secondary metabolites, altered strain characteristics and/or conditional lysis, or increased resistance to the deleterious effects of exposure to a secondary metabolite.

The several aspects of the methods according to the invention are preferably achieved by overexpression of regulatory genes, expression of dominant mutations, use of peptide activators or inhibitors, use of small molecule activators or inhibitors, and conditional expression of regulatory genes. These factors preferably modulate transcription factors, transmembrane transporters, proteins that mediate secretion, kinases, G-proteins, cell surface receptors, GTPase activating proteins, guanine nucleotide exchange factors, phosphatases, proteases, phosphodiesterases, bacterial protein toxins, importins, RNA-binding proteins, SCF complex components, adherins, or biosynthetic pathways.

The invention further provides for achieving the aspects described in the invention by combinatorial manipulation. Combinatorial manipulation is the simultaneous use of multiple methods and/or multiple factors to achieve the aspects of the invention. Methods for achieving the aspects of the invention are preferably by the overexpression of regulatory genes, expression of dominant mutations, use of peptide activators or inhibitors, use of small molecule activators or inhibitors, and conditional expression of regulatory genes. The preferred factors are as described above.

The invention further provides genetically modified fungi, wherein the genetically modified fungi have an ability to produce secondary metabolites and the ability of the genetically modified fungus to produce secondary metabolites has been improved by any of the methods according to the invention.

The invention also provides a method for making a secondary metabolite, the method comprising culturing a genetically modified fungus according to the invention under conditions suitable for the production of secondary metabolites.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the ability of PUMP1 (AAD34558) from *Aspergillus terreus* to confer lovastatin resistance to a yeast strain.

Figure 2 shows the impact of yeast genetics and genomics on fungal genetics. Arrows indicate which genes or gene products act on other genes or gene products.

Figure 3 shows representative box plot presentations of lovastatin production data from shake flask experiments. Data from strains that express a particular regulator (*e.g.*, pacCVP16) are displayed with appropriate negative (EMPTY or GUS) and positive (lovE) controls from the same shake flask experiment.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to the production of secondary metabolites by fungi. More particularly, the invention relates to improvement of production of commercially important secondary metabolites by fungi. The references cited herein evidence the level of knowledge in the field and are therefore incorporated by reference in their entirety. In the event of a conflict between a cited reference and the present specification, the latter shall prevail.

The invention provides methods for improving secondary metabolite production in a fungus, comprising modulating the expression of a gene involved in regulation of secondary metabolite production. In certain embodiments, the methods comprise modulating the expression of more than one gene involved in regulation of secondary metabolite production.

The experiments disclosed in this specification demonstrate how genetic manipulation can be employed to improve the process of secondary metabolite production in fungi. In these experiments, strains have been manipulated to express fungal regulators, and in many instances these modifications resulted in improvements such as increased yield of metabolite, increased productivity of metabolite, or beneficial morphological and behavioral characteristics. These manipulations have improved production of secondary metabolites, including both the β-lactam antibiotic penicillin as well as the polyketide anti-hypercholesterolemic drug lovastatin. The discovery that this engineering (of regulatory pathways instead of biosynthetic genes) can improve the production of multiple classes of drugs together with the demonstration that this process can be implemented in various fungi suggests that the process of genetic manipulation of regulators of secondary metabolism will be a general tool for improving secondary metabolite production in fungi.

In a first aspect, the invention provides methods for improving production of a secondary metabolite by a fungus by increasing the yield of the secondary metabolite produced by the fungus. The methods according to this aspect of the invention comprise modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that improves the yield of the secondary metabolite.

Preferably, for this aspect of the invention, when the secondary metabolite is isopenicillin N, then the modulation is not mediated by the transcription factor CPCR1; when the secondary metabolite is sterigmatocystin, then the modulation is not through AflR, FadA, or FluG; when the secondary metabolite is aflatoxin, then the modulation is not through AflR; when the secondary metabolite is penicillin and the fungus is *Aspergillus nidulans*, then the modulation is not through mutations that result in expression of truncated forms of PacC or constitutively active forms of FadA; when the gene involved in regulation of secondary metabolite production is from *Saccharomyces cerevisiae*, then the modulation is not through decreased activity or expression of BEM2, HOG1, IRA1, RIM15, SFL1, SRB11, SSD1, SWI4, TPK3 or though increased activity or expression of AFL1, CDC25, DHH1, HAP4, INV11, INV13, INV5, INV7, INV9, MCM1, MEP2, MGA1, MSN1, MSN5, MSS11, PET9, PHO23, PTC1, RIM101, RIM13, RIM9, SNF8, STP22, TPK2, VPS28, VPS36, or YPR1.

As used for all aspects of the invention, the term "improving production of a secondary metabolite" means to positively impact upon one or more of the variables that affect the process of production of secondary metabolites in a fungal fermentation. These variables include, without limitation, amount of secondary metabolite being produced, the volume required for production of sufficient quantities, the cost of raw materials and energy, the time of fermentor run, the amount of waste that must be processed after a fermentor run, the specific production of the desired metabolite, the percent of produced secondary metabolite that can be recovered from the fermentation broth, and the resistance of an organism producing a secondary metabolite to possible deleterious effects of contact with the secondary metabolite. Also for all aspects, the term "secondary metabolite" means a compound, derived from primary metabolites, that is produced by an organism, is not a primary metabolite, is not ethanol or a fusel alcohol, and is not required for growth under standard conditions. Secondary metabolites are derived from intermediates of many pathways of primary metabolism. These pathways include, without limitation, pathways for biosynthesis of amino acids, the shikimic acid pathway for biosynthesis of aromatic amino acids, the polyketide biosynthetic pathway from acetyl coenzyme A (CoA), the mevalonic acid pathway from acetyl CoA, and pathways for biosynthesis of polysaccharides and peptidopolysaccharides. Secondary metabolism involves all primary pathways of carbon metabolism (Fungal Physiology, Chapter 9 pp. 246-274 ed. DH Griffin (1994)). "Secondary

metabolites" also include intermediate compounds in the biosynthetic pathway for a secondary metabolite that are dedicated to the pathway for synthesis of the secondary metabolite. "Dedicated to the pathway for synthesis of the secondary metabolite" means that once the intermediate is synthesized by the cell, the cell will not convert the intermediate to a primary metabolite. "Intermediate compounds" also include secondary metabolite intermediate compounds which can be converted to useful compounds by subsequent chemical conversion or subsequent biotransformation. Nevertheless, providing improved availability of such intermediate compounds would still lead to improved production of the ultimate useful compound, which itself may be referred to herein as a secondary metabolite. The yeast *Saccharomyces cerevisiae* is not known to produce secondary metabolites. The term "primary metabolite" means a natural product that has an obvious role in the functioning of almost all organisms. Primary metabolites include, without limitation, compounds involved in the biosynthesis of lipids, carbohydrates, proteins, and nucleic acids. The term "increasing the yield of the secondary metabolite" means increasing the quantity of the secondary metabolite present in the fermentation broth per unit volume of fermentation broth.

A "gene involved in regulation of secondary metabolite production" is a gene, other than a gene encoding a biosynthetic enzyme, which modulates secondary metabolite production involving yield, productivity, efflux/excretion, production of side products or non-desired secondary metabolites, strain characteristics and/or conditional lysis, or resistance to the deleterious effects of exposure to a secondary metabolite. A "biosynthetic enzyme" is a molecule that catalyzes the conversion of a substrate to a product, including an intermediate product, in a biosynthetic pathway for a secondary metabolite.

As used for all aspects of the invention, the term "modulating the expression of a gene" means affecting the function of a gene's product, preferably by increasing or decreasing protein activity through mutation, creating a new protein activity through mutation; increasing or decreasing transcription, increasing or decreasing translation, increasing or decreasing post-translational modification, altering intracellular localization, increasing or decreasing translocation, increasing or decreasing protein activity by interaction of the protein with another molecule, or creating a new protein activity by interaction of the protein with another molecule. In some cases, such modulation is achieved simply by allowing or causing the expression of an

exogenously supplied nucleic acid or gene. In some cases other exogenously supplied molecules may mediate the modulation. The modulation is not achieved, however, by simply randomly mutagenizing the fungus, either spontaneously or by chemical means.

As used for all aspects of the invention, "mutation" means an alteration in DNA sequence, either by site-directed or random mutagenesis or by recombination. Mutation encompasses point mutations as well as insertions, deletions, or rearrangements.

As used for all aspects of the invention, "mutant" means an organism containing one or more mutations.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" means transcription and/or translation and/or gene product maturation at a rate that exceeds by at least two-fold, preferably at least five fold, and more preferably at least ten-fold, the level of such expression that would be present under similar growth conditions in the absence of the modulation of expression of the gene. "Similar growth conditions" means similar sources of nutrients such as carbon, nitrogen, and phosphate, as well as similar pH, partial oxygen pressure, temperature, concentration of drugs or other small molecules, and a similar substrate for growth, whether solid, semi-solid, or liquid. Preferred genes according to this aspect of the invention include, without limitation, AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3,

YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. A "dominant mutation" is an allele of a gene that encodes a protein capable of changing the phenotype of an organism more than a non-mutated form of the gene. Preferred dominant mutations include dominant negative mutations, dominant positive mutations, and dominant neomorphic mutations. A "dominant negative mutation" is a dominant mutation that achieves its phenotypic effect by interfering with some function of the gene or gene product from which it was derived, or from a homolog thereof. A "dominant positive mutation" is a dominant mutation that achieves its phenotypic effect by activating some function of the gene or gene product from which it was derived, or from a homolog thereof. A "dominant neomorphic mutation" is a dominant mutation that achieves the phenotypic effect of providing a novel function to the gene or gene product from which it was derived, or from a homolog thereof. Preferred dominant mutations according to this aspect of the invention include:

1. Mutations that result in increased or decreased stability of the transcript of a gene.
2. Mutations that result in increased or decreased stability of the product of translation: For example, specific sequences near the amino terminus of a protein have been shown to cause increased or decreased protein stability. Similarly, sequences elsewhere in the protein, such as those required for ubiquitin-dependent degradation, can be mutated to increase the stability of a protein.
3. Amino acid substitutions that mimic post-translational modifications: For example, phosphorylation has been demonstrated to positively or negatively regulate the activity of a variety of proteins, including transcription factors and kinases. Phosphorylation most commonly occurs on serine, threonine, and tyrosine residues; in some instances residues such as aspartate and histidine can be phosphorylated. Mutations that mimic constitutive dephosphorylation can be produced by mutating the coding sequence of the phosphorylated residue to the coding sequence of an amino acid that cannot be phosphorylated and does not have a negatively charged side chain (e.g. alanine). Alternatively, substitutions that result in changing serine, threonine, or

tyrosine residues to charged amino acids such as glutamate or aspartate can result in an allele that mimics constitutive phosphorylation.

Proteolytic cleavage is another post-translation mechanism for regulating the activity of a protein. Mutations that result in truncation of a protein might mimic activation by proteolysis. Mutations that change amino acids required for proteolysis could activate proteins that are negatively regulated by proteolysis.

4. Amino acid substitutions that promote or inhibit the binding of small molecules such as ATP, cAMP, GTP or GDP: For example, ATP is a co-factor for many enzymatic reactions, and the nucleotide-binding domains of these proteins are highly conserved. Lysine to arginine substitutions in the nucleotide binding domain frequently result in inhibition of enzymatic activity. Enzymatically inactive proteins could be dominant inactive molecules, acting by sequestering substrates from functional enzymes.

cAMP is required for the activation of protein kinase A. Protein kinase A consists of regulatory subunits and catalytic subunits. The binding of cAMP to the negative regulatory subunit relieves its inhibition of the catalytic subunit. Therefore, mutations that prevent cAMP binding could result in constitutive inactivation of protein kinase A.

G-proteins are a class of proteins that bind the nucleotides GTP and GDP. The GTP-bound form of these proteins is active, and hydrolysis of GTP to GDP results in the inactivation of the protein. Conserved substitutions can be made to lock G-proteins in either the GTP- or GDP-bound form, thus causing constitutive activation or inactivation.

5. Mutations in portions of genes that encode regulatory domains of proteins: For example, many proteins, including kinases, contain regulatory domains that function as mechanisms to control the timing of activation. Mutations in these domains might result in the constitutive activation. Mutations that result in increased binding to regulatory proteins might result in constitutive inactivation.

Regulatory domains include short peptide sequences such as those required for nuclear import or export. Mutations in these sequences would result in constitutive

cytoplasmic or nuclear localization, respectively, which could either activate or inhibit signaling.

6. Mutations that result in proteins that are capable of binding to an appropriate signaling partner, but the complexes that form are inactive: For example, dimerization of proteins, either homodimers or heterodimers, often is required for signaling; in many instances, short protein sequences are sufficient to promote dimerization. Mutations in functional domains not required for dimerization might result in dominant inhibition; these proteins are capable of binding to and possibly sequestering other signaling molecules into inactive, or partially inactive, complexes.
7. Mutations that decrease or increase the targeting of proteins to the appropriate subcellular destination: Short peptide sequences often facilitate the targeting of proteins to specific subcellular locations. For example, short sequences are sufficient to be recognized and modified by fatty acylation, prenylation, or glycosyl-phosphatidylinositol modification. These modifications result in targeting of proteins to membranes. Membrane spanning peptide sequences also have been identified, as have targeting sequences for secretion. In addition, sequences have been identified that target proteins to subcellular locations such as the endoplasmic reticulum, mitochondria, peroxisome, vacuole, nucleus, and lysosome. Mutations that inhibit proper targeting might result in dominant inhibition; these proteins might be capable of binding to and possibly sequestering other signaling molecules from the appropriate subcellular location.
8. Mutations that create a new protein function. For example, a mutation in a protein kinase could result in altered substrate specificity, such that the mutated kinase can modulate the activity of pathways that it does not usually regulate.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" means a molecule comprised of a linear array of amino acid residues connected to each other in the linear array by peptide bonds. Such peptides according to the invention may include from about three to about 500 amino acids, and may further include secondary, tertiary or quaternary structures, as

well as intermolecular associations with other peptides or other non-peptide molecules. Such intermolecular associations may be through, without limitation, covalent bonding (*e.g.*, through disulfide linkages), or through chelation, electrostatic interactions, hydrophobic interactions, hydrogen bonding, ion-dipole interactions, dipole-dipole interactions, or any combination of the above. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. A "scaffold" is a molecule, most frequently a small protein, from which a peptide is displayed; scaffolds are employed to optimize presentation, rigidity, conformational constraint, and potentially intracellular/extracellular localization. Preferred scaffolds include a catalytically inactive version of staphylococcal nuclease. Preferred peptides according to this aspect of the invention include, without limitation, those peptides disclosed in Norman *et al.*, Science 285: 591-595 (1999).

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. An "activator of gene expression" is a molecule that causes transcription and/or translation and/or gene product maturation to exceed by at least two-fold, preferably at least five fold, and more preferably at least ten-fold, the level of such expression that would be present under similar growth conditions in the absence of the activator of expression of the gene. "Similar growth conditions" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. An "inhibitor of gene expression" is a molecule that causes transcription and/or translation and/or gene product maturation to be reduced by at least two-fold, preferably at least five fold, and more preferably at least ten-fold, the level of such expression that would be present under similar growth conditions in the absence of the inhibitor of expression of the gene. "Similar growth conditions" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene

expression" is as used before. A "small molecule" is a compound with a preferable molecular weight below 1000 daltons.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. "Conditional expression" of a gene means expression under certain growth conditions, but not under others. Such growth conditions that may be varied include, without limitation, carbon source, nitrogen source, phosphate source, pH, temperature, partial oxygen pressure, the presence or absence of small molecules such as drugs, and the presence or absence of a solid substrate.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. As used throughout this specification, the term "the gene acts on" means that the gene or its transcriptional, translational, or post-translationally modified product affects the function of its target (the word following the expression "the gene acts on"), preferably by increasing or decreasing transcription, increasing or decreasing translation, increasing or decreasing post-translational modification, increasing or decreasing protein stability, increasing or decreasing protein translocation, or increasing or decreasing protein function by interaction of the protein with another molecule. A "transcription factor" is a molecule that activates or inhibits transcription. The term "activates transcription" means to cause transcription to exceed by at least two-fold, preferably at least five fold, and more preferably at least ten-fold, the level of transcription that would be present under similar growth conditions in the absence of the transcription factor. The term "inhibits transcription" means to cause transcription to be reduced by at least two-fold, preferably at least five fold, and more preferably at least ten-fold, the level of such transcription that would be present under similar growth conditions in the absence of the transcription factor. Preferred transcription factors include, without limitation, transcription factors that modulate the expression of genes involved in the production or response to the small molecule cAMP (preferred examples include, without limitation, MGA1, MSN2, MSN4, SFL1, and SOK2); transcription factors that function downstream of mitogen-activated protein (MAP) kinase signaling pathways that regulate the yeast invasion response (preferred examples include, without limitation, MCM1, STE12, and TEC1); transcription factors that modulate the expression of genes involved in nitrogen regulation (preferred examples include, without

limitation, areA, GLN3, HMS1, HMS2, nreB, tamA, and UGA3); transcription factors that modulate the expression of genes involved in pH regulation in fungi (preferred examples include, without limitation pacC and RIM101); general transcription factors (preferred examples include, without limitation, SIN3, SNF2, SRB8, SRB9, SRB10, SRB11, SSN6, and TUP1); transcription factors that modulate the expression of genes involved in carbon metabolism (preferred examples include, without limitation, ADR1, CAT8, creA, facB, GCR1, GCR2, HAP4, MIG1, MIG2, MTH1, NRG1, OAF1, and SIP4); heme-dependent transcription factors (preferred examples include, without limitation, HAP1 and ROX1); transcription factors that modulate the expression of genes involved in the uptake of metals (preferred examples include, without limitation, AFT1, CUP9, MAC1, sreP, sreA, and ZAP1); transcription factors that modulate the expression of genes involved in cell-cycle regulation (preferred examples include, without limitation, SKN7, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in invasion (preferred examples include, without limitation, ASH1, FLO8, GTS1, INV7, MSN1, MSS11, PHD1, and RRE1); transcription factors that modulate the expression of genes involved in amino acid biosynthesis or transport (preferred examples include, without limitation, GCN4, LEU3, LYS14, MET4, MET28, MET31, metR, PUT3, sconB, and UGA3); transcription factors that modulate the expression of genes involved in phosphate metabolism or transport (preferred examples include, without limitation, PHO2 and PHO4); transcription factors that modulate the expression of genes involved in nucleotide metabolism or transport (preferred examples include, without limitation, PPR1 and UaY); transcription factors that modulate the expression of genes involved in cell wall processes (preferred examples include, without limitation, ACE2, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in sporulation (preferred examples include, without limitation, IME1 and IME4); transcription factors that modulate the expression of genes involved in phospholipid synthesis (preferred examples include, without limitation, INO2); transcription factors that modulate the expression of genes involved in aflatoxin biosynthesis (preferred examples include, without limitation, aflR); transcription factors that modulate the expression of genes involved in lovastatin biosynthesis (preferred examples include, without limitation, AAD34561 and lovE); and transcription factors that modulate the expression of genes involved in filamentous fungal development (preferred examples include, without limitation, abaA). The term "general transcription factors" means

components involved in the formation of preinitiation complexes at promoters that are regulated by RNA polymerase II. The term "invasion" means a process by which a fungus penetrates, digs, adheres to, or attaches to a substrate.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. A "transmembrane transporter" is a molecule or complex of molecules that facilitates passage of another type of molecule from one side of a cellular membrane to the other side in an energy-dependent or energy-independent manner. "Facilitates passage" means that the number of molecules traversing the membrane is greater than it would have been in the absence of the transmembrane pump, preferably at least two-fold greater, more preferably at least ten-fold greater, even more preferably at least one hundred-fold greater, and most preferably at least one thousand-fold greater. Preferred classes of transmembrane transporters include, without limitation, proteins of the ATP-binding cassette superfamily, members of the Major Facilitator Superfamily (MFS), P-type ATPases, members of the mitochondrial carrier family (MCF) that include, without limitation, PET9; ion channels, permeases that include, without limitation, BAP2, HIP1, MEP1, and MEP2; and components that transport sugars, ions, or metals.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. A "kinase" is a molecule that phosphorylates a protein, a lipid, a nucleic acid, a carbohydrate, or any other substrate that is capable of being phosphorylated. Preferred kinases include, without limitation, CDC28, ELM1, FUS3, GCN2, HOG1, HSL1, HXK2, KSS1, PBS2, PHO85, RIM15, STE7, SCH9, SNF1, STE11, STE20, TPK1, TPK2, and TPK3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. A "G-protein" is a guanyl-nucleotide binding protein. Preferred G-proteins include, without limitation CDC42, fadA, GPA1, GPA2, RAS1, and RAS2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. A "cell surface receptor" is a molecule that resides at the plasma membrane, binds an

extracellular signaling molecule, and transduces this signal to propagate a cellular response. Preferred cell surface receptors include, without limitation, G-protein coupled receptors. Preferred G-protein coupled receptors include, without limitation, GPR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. A "GTPase activating protein" is a molecule that promotes the hydrolysis of GTP bound to a G-protein. GTP-activating proteins often negatively regulate the activity of G-proteins. Preferred GTPase activating proteins include, without limitation, RGS family members. "RGS family members" are regulators of G-protein signaling that act upon G-protein coupled receptors. Preferred RGS family members include, without limitation, FlbA, RGS2, and SST2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. A "guanine nucleotide exchange factor" is a molecule that catalyzes the dissociation of GDP from the inactive GTP-binding proteins; following dissociation, GTP can then bind and induce structural changes that activate G-protein signaling. Preferred guanine nucleotide exchange factors include, without limitation, CDC24 and CDC25.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. A "phosphatase" is a molecule that dephosphorylates a protein, a lipid, a nucleic acid, a carbohydrate, or any other substrate that is capable of being dephosphorylated. Preferred phosphatases include, without limitation, CDC55 and PTC1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. A "protease" is a molecule that cleaves an amide bond in a peptide. "Peptide" is as used before. Preferred proteases include, without limitation, RIM13.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. A "cyclic nucleotide phosphodiesterase" is a molecule catalyzes the hydrolysis of the 3' phosphate bond of a 3', 5' cyclic nucleotide to yield free 5'

nucleotide. Preferred examples of cyclic nucleotide phosphodiesterases include, without limitation, PDE2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. A "bacterial protein toxin" is protein produced by a bacterium, as part of the pathogenesis of the bacterial organism, to kill or impair the biological function of the host organism. Bacterial protein toxins exhibit a wide-variety of biochemical and enzymatic activities including those of adenylate cyclases, ADP-ribosyltransferases, phospholipases, and proteases. Expression of bacterial protein toxins in fungi could result in increased production of secondary metabolites. Preferred bacterial protein toxins include, without limitation, Anthrax toxin edema factor (EF; *Bacillus anthracis*), Anthrax toxin lethal factor (LF; *Bacillus anthracis*), adenylate cyclase toxin (*Bordetella pertussis*), Cholera enterotoxin (*Vibrio cholerae*), LT toxin (*Escherichia coli*), ST toxin (*E. coli*), Shiga toxin (*Shigella dysenteriae*), Perfringens enterotoxin (*Clostridium perfringens*), Botulinum toxin (*Clostridium botulinum*), Tetanus toxin (*Clostridium tetani*), Diphtheria toxin (*Corynebacterium diphtheriae*), Exotoxin A (*Pseudomonas aeruginosa*), Exoenzyme S (*P. aeruginosa*), Pertussis toxin (*Bordetella pertussis*), alpha and epsilon toxins (*C. perfringens*), lethal toxin (LT; *Clostridium sordellii*), toxins A and B (*Clostridium dificile*), and phospholipase C (*Clostridium bifermentans*).

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on a importin protein. An "importin" protein is a molecule that functions in the translocation of proteins from the nucleus to the cytosol or from the cytosol from the nucleus. Preferred examples of importin proteins include, without limitation, MSN5.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an RNA-binding protein or the product that it encodes acts on an RNA-binding protein. Preferred examples of RNA-binding proteins include, without limitation, DHH1 and WHI3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. A "component of a SCF complex" is a molecule in a multi-protein aggregate

that targets various substrates involved in the G1 to S phase cell cycle transition for ubiquitin-dependent degradation. Preferred examples of components of a SCF complex include, without limitation, GRR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. An "anti-bacterial" is a molecule that has cytocidal or cytostatic activity against some or all bacteria. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is a drug administered to a patient diagnosed with elevated cholesterol levels, for the purpose of lowering the cholesterol levels. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is a molecule that reduces or eliminates an immune response in a host when the host is challenged with an immunogenic molecule, including immunogenic molecules present on transplanted organs, tissues or cells. Preferred immunosuppressants include, without limitation, members of the

cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. An "ergot alkaloid" is a member of a large family of alkaloid compounds that are most often produced in the sclerotia of fungi of the genus Claviceps. An "alkaloid" is a small molecule that contains nitrogen and has basic pH characteristics. The classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. An "angiogenesis inhibitor" is a molecule that decreases or prevents the formation of new blood vessels. Angiogenesis inhibitors have proven effective in the treatment of several human diseases including, without limitation, cancer, rheumatoid arthritis, and diabetic retinopathy. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. A "glucan synthase inhibitor" is a molecule that decreases or inhibits the production of 1,3-B-D-glucan, a structural polymer of fungal cell walls. Glucan synthase inhibitors are a class of antifungal agents. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The "gliotoxin family of compounds" are related molecules of the epipolythiodioxopiperazine class. Gliotoxins display diverse biological activities, including, without limitation, antimicrobial, antifungal, antiviral, and immunomodulating activities. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. A "fungal toxin" is a compound that causes a

pathological condition in a host, either plant or animal. Fungal toxins could be mycotoxins present in food products, toxins produced by phytopathogens, toxins from poisonous mushrooms, or toxins produced by zoopathogens. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Modulators of cell surface receptor signaling might function by one of several mechanisms including, without limitation, acting as agonists or antagonists, sequestering a molecule that interacts with a receptor such as a ligand, or stabilizing the interaction of a receptor and molecule with which it interacts. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. A "plant growth regulator" is a molecule that controls growth and development of a plant by affecting processes that include, without limitation, division, elongation, and differentiation of cells. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. A "pigment" is a substance that imparts a characteristic color. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. An "insecticide" is a molecule that is toxic to insects. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. An "anti-neoplastic" compound is a molecule that prevents or reduces tumor formation. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is not AFL1, BEM2, CDC25, DHH1, HOG1, INV11, INV13, INV5, INV7, INV9, IRA1, MCM1, MEP2, MGA1, MSN1, MSN5, MSS11, PET9, PHO23, PTC1, RIM101, RIM13, RIM15, RIM9, SFL1, SNF8, SRB11, SSD1, STP22, SWI4, TPK2, TPK3, VPS28, VPS36, or YPR1. Each of these genes is as described in PCT Publication No. WO99/25865A1

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes. A "fungal homolog" of a gene is a gene encoding a gene product that is capable of performing at least a portion of the function of the product encoded by the reference gene, and is substantially identical to the reference gene and/or the encoded product. "Substantially identical" means a polypeptide or nucleic acid exhibiting at least 25%, preferably 50%, more preferably 80%, and most preferably 90%, or even 95% identity to a reference amino acid sequence or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids or greater. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides,

preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides or greater.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison WI 53705, BLAST, BEAUTY, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following group: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. "Purifying" means obtaining the secondary metabolite in substantially pure form. "Substantially pure" means comprising at least 90 %, more preferably at least 95 %, and most preferably at least 99 %, of the purified composition on a weight basis.

In a second aspect, the invention provides methods for improving production of a secondary metabolite by a fungus by increasing productivity of the secondary metabolite in the fungus, the methods comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that improves the productivity of the secondary metabolite. "Increasing productivity" means to increase the quotient for the equation concentration of secondary metabolite divided by the product of time of fermentor run, fermentation volume, and grams of dry cell weight of biomass (Productivity= concentration metabolite/ (time*volume*gDCW)). Significant advantages that might result from increasing productivity include, without limitation, a decrease in fermentor run time, a decrease in the size of fermentor required for production of equivalent amounts of secondary metabolite, or a decrease in the biomass required for production, which translates into decrease waste that must be handled in downstream processing. Preferably, such increased productivity is by at least ten percent, more preferably at least 50 percent, and most preferably at least two-fold.

Preferably, for this aspect of the invention, when the secondary metabolite is isopenicillin N, then the modulation is not mediated by the transcription factor CPCR1; when the secondary metabolite is sterigmatocystin, then the modulation is not through AflR, FadA, or FluG; when the secondary metabolite is aflatoxin, then the modulation is not through AflR; when the secondary metabolite is penicillin and the fungus is Aspergillus, then the modulation is not through mutations that result in expression of truncated forms of PacC or constitutively active forms of FadA; when the gene involved in regulation of secondary metabolite production is from *Saccharomyces cerevisiae*, then the modulation is not through decreased activity or expression of BEM2, HOG1, IRA1, RIM15, SFL1, SRB11, SSD1, SWI4, TPK3 or though increased activity or expression of AFL1, CDC25, DHH1, HAP4, INV11, INV13, INV5, INV7, INV9, MCM1, MEP2, MGA1, MSN1, MSN5, MSS11, PET9, PHO23, PTC1, RIM101, RIM13, RIM9, SNF8, STP22, TPK2, VPS28, VPS36, or YPR1.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is

as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. The term "the gene acts on" is as used before. The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, transcription factors that modulate the expression of genes involved in the production or response to the small molecule cAMP (preferred examples include, without limitation, MGA1, MSN2, MSN4, SFL1, and SOK2); transcription factors that function downstream of mitogen-activated protein (MAP) kinase

signaling pathways that regulate the yeast invasion response (preferred examples include, without limitation, MCM1, STE12, and TEC1); transcription factors that modulate the expression of genes involved in nitrogen regulation (preferred examples include, without limitation, areA, GLN3, HMS1, HMS2, nreB, tamA, and UGA3); transcription factors that modulate the expression of genes involved in pH regulation in fungi (preferred examples include, without limitation pacC and RIM101); general transcription factors (preferred examples include, without limitation, SIN3, SNF2, SRB8, SRB9, SRB10, SRB11, SSN6, and TUP1); transcription factors that modulate the expression of genes involved in carbon metabolism (preferred examples include, without limitation, ADR1, CAT8, creA, facB, GCR1, GCR2, HAP4, MIG1, MIG2, MTH1, NRG1, OAF1, and SIP4); heme-dependent transcription factors (preferred examples include, without limitation, HAP1 and ROX1); transcription factors that modulate the expression of genes involved in the uptake of metals (preferred examples include, without limitation, AFT1, CUP9, MAC1, sreP, sreA, and ZAP1); transcription factors that modulate the expression of genes involved in cell-cycle regulation (preferred examples include, without limitation, SKN7, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in invasion (preferred examples include, without limitation, ASH1, FLO8, GTS1, INV7, MSN1, MSS11, PHD1, and RRE1); transcription factors that modulate the expression of genes involved in amino acid biosynthesis or transport (preferred examples include, without limitation, GCN4, LEU3, LYS14, MET4, MET28, MET31, metR, PUT3, sconB, and UGA3); transcription factors that modulate the expression of genes involved in phosphate metabolism or transport (preferred examples include, without limitation, PHO2 and PHO4); transcription factors that modulate the expression of genes involved in nucleotide metabolism or transport (preferred examples include, without limitation, PPR1 and UaY); transcription factors that modulate the expression of genes involved in cell wall processes (preferred examples include, without limitation, ACE2, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in sporulation (preferred examples include, without limitation, IME1 and IME4); transcription factors that modulate the expression of genes involved in phospholipid synthesis (preferred examples include, without limitation, INO2); transcription factors that modulate the expression of genes involved in aflatoxin biosynthesis (preferred examples include, without limitation, aflR); transcription factors that modulate the expression of genes involved in lovastatin biosynthesis

(preferred examples include, without limitation, AAD34561 and lovE); and transcription factors that modulate the expression of genes involved in filamentous fungal development (preferred examples include, without limitation, abaA). The term "general transcription factors" is as used before. The term "invasion" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. The term "transmembrane transporter" is as used before. Preferred classes of transmembrane transporters include, without limitation, proteins of the ATP-binding cassette superfamily, members of the Major Facilitator Superfamily (MFS), P-type ATPases, members of the mitochondrial carrier family (MCF) that include, without limitation, PET9; ion channels, permeases that include, without limitation, BAP2, HIP1, MEP1, and MEP2; and components that transport sugars, ions, or metals.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. The term "kinase" is as used before. Preferred kinases include, without limitation, CDC28, ELM1, FUS3, GCN2, HOG1, HSL1, HXK2, KSS1, PBS2, PHO85, RIM15, STE7, SCH9, SNF1, STE11, STE20, TPK1, TPK2, and TPK3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. The term "G-protein" is as used before. Preferred G-proteins include, without limitation CDC42, fadA, GPA1, GPA2, RAS1, and RAS2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. The term "cell surface receptor" is as used before. Preferred cell surface receptors include, without limitation, G-protein coupled receptors. Preferred G-protein coupled receptors include, without limitation, GPR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. The term "GTPase activating protein" is as used before. Preferred GTPase activating proteins include, without limitation, RGS family members. "RGS family members"

are regulators of G-protein signaling that act upon G-protein coupled receptors. Preferred RGS family members include, without limitation, FlbA, RGS2, and SST2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. The term "guanine nucleotide exchange factor" is as used before. Preferred guanine nucleotide exchange factors include, without limitation, CDC24 and CDC25.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. The term "phosphatase" is as used before. Preferred phosphatases include, without limitation, CDC55 and PTC1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. The term "protease" is as used before. Preferred proteases include, without limitation, RIM13.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. The term "cyclic nucleotide phosphodiesterase" is as used before. Preferred examples of cyclic nucleotide phosphodiesterases include, without limitation, PDE2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. The term "bacterial protein toxin" is as used before. Preferred bacterial protein toxins include, without limitation, Anthrax toxin edema factor (EF; *Bacillus anthracis*), Anthrax toxin lethal factor (LF; *Bacillus anthracis*), adenylate cyclase toxin (*Bordetella pertussis*), Cholera enterotoxin (*Vibrio cholerae*), LT toxin (*Escherichia coli*), ST toxin (*E. coli*), Shiga toxin (*Shigella dysenteriae*), Perfringens enterotoxin (*Clostridium perfringens*), Botulinum toxin (*Clostridium botulinum*), Tetanus toxin (*Clostridium tetani*), Diphtheria toxin (*Corynebacterium diphtheriae*), Exotoxin A (*Pseudomonas aeruginosa*), Exoenzyme S (*P. aeruginosa*), Pertussis toxin (*Bordetella pertussis*), alpha and epsilon toxins (*C. perfringens*), lethal toxin (LT;

Clostridium sordellii), toxins A and B (*Clostridium dificile*), and phospholipase C (*Clostridium bifermentans*).

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on a importin protein. The term "importin" protein is as used before. Preferred examples of importin proteins include, without limitation, MSN5.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an RNA-binding protein or the product that it encodes acts on an RNA-binding protein. Preferred examples of RNA-binding proteins include, without limitation, DHH1 and WHI3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. The term "component of a SCF complex" is as used before. Preferred examples of components of a SCF complex include, without limitation, GRR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is as used

before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin,

bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB,

NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, sconB, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes. The term "fungal homolog" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a third aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by increasing efflux or excretion of the secondary metabolite, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that increases efflux or excretion of the secondary metabolite. "Increasing efflux or excretion of the secondary metabolite" means that a greater quantity of the secondary metabolite passes from the inside of the fungal cells to the outside of the fungal cell per unit time in the absence of lysis of the fungal cells. "Outside of the fungal cell" is defined as being no longer contained wholly within the lipid bilayer of the cell and/or extractable from the cell with methods which do not release a majority of intracellular contents.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, AAD34558, AAD34561, AAD34564, ATR1, ERG6, FCR1, GCN4, lovE, MDR1, PDR1, PDR3, PDR5, PDR10, PDR13, SNQ2, TRI12, and YAP1.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, AAD34561, FCR1, GCN4, lovE, PDR1, PDR3, and YAP1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. The term "transmembrane transporter" is as used before. Preferred transmembrane transporters include, without limitation, AAD34558, AAD34564, ATR1, MDR1, PDR5, PDR10, SNQ2, and TRI12.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. A "kinase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. A "G-protein" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. A "cell surface receptor" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. A "GTPase activating protein" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. A "guanine nucleotide exchange factor" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. A "phosphatase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. A "protease" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. A "cyclic nucleotide phosphodiesterase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. A "bacterial protein toxin" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on a importin protein. An "importin" protein is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an RNA-binding protein or the product that it encodes acts on an RNA-binding protein.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. A "component of a SCF complex" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is as used before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred

classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used

before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34558, AAD34561, AAD34564, ATR1, ERG6, FCR1, GCN4, lovE, MDR1, PDR1, PDR3, PDR5, PDR10, PDR13, SNQ2, TRI12, YAP1, and any fungal homologs of the aforementioned genes. The term "fungal homolog" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a fourth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by decreasing production of side products or non-desired secondary metabolites, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that decreases production of side products or non-desired secondary metabolites.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3,

ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used

before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. The term "the gene acts on" is as used before. The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, transcription factors that modulate the expression of genes involved in the production or response to the small molecule cAMP (preferred examples include, without limitation, MGA1, MSN2, MSN4, SFL1, and SOK2); transcription factors that function downstream of mitogen-activated protein (MAP) kinase signaling pathways that regulate the yeast invasion response (preferred examples include, without limitation, MCM1, STE12, and TEC1); transcription factors that modulate the expression of genes involved in nitrogen regulation (preferred examples include, without limitation, areA, GLN3, HMS1, HMS2, nreB, tamA, and UGA3); transcription factors that modulate the expression of genes involved in pH regulation in fungi (preferred examples include, without limitation pacC and RIM101); general transcription factors (preferred examples include, without limitation, SIN3, SNF2, SRB8, SRB9, SRB10, SRB11, SSN6, and TUP1); transcription factors that modulate the expression of genes involved in carbon metabolism (preferred examples include, without limitation, ADR1, CAT8, creA, facB, GCR1, GCR2, HAP4, MIG1, MIG2, MTH1, NRG1, OAF1, and SIP4); heme-dependent transcription factors (preferred examples include, without limitation, HAP1 and ROX1); transcription factors that modulate the expression of genes involved in the uptake of metals (preferred examples include, without limitation, AFT1, CUP9, MAC1, sreP, sreA, and ZAP1); transcription factors that modulate the expression of genes involved in cell-cycle regulation (preferred examples include, without limitation, SKN7, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in invasion (preferred examples include, without limitation, ASH1, FLO8, GTS1, INV7, MSN1, MSS11, PHD1, and RRE1); transcription factors that modulate the expression of genes involved

in amino acid biosynthesis or transport (preferred examples include, without limitation, GCN4, LEU3, LYS14, MET4, MET28, MET31, metR, PUT3, sconB, and UGA3); transcription factors that modulate the expression of genes involved in phosphate metabolism or transport (preferred examples include, without limitation, PHO2 and PHO4); transcription factors that modulate the expression of genes involved in nucleotide metabolism or transport (preferred examples include, without limitation, PPR1 and UaY); transcription factors that modulate the expression of genes involved in cell wall processes (preferred examples include, without limitation, ACE2, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in sporulation (preferred examples include, without limitation, IME1 and IME4); transcription factors that modulate the expression of genes involved in phospholipid synthesis (preferred examples include, without limitation, INO2); transcription factors that modulate the expression of genes involved in aflatoxin biosynthesis (preferred examples include, without limitation, aflR); transcription factors that modulate the expression of genes involved in lovastatin biosynthesis (preferred examples include, without limitation, AAD34561 and lovE); and transcription factors that modulate the expression of genes involved in filamentous fungal development (preferred examples include, without limitation, abaA). The term "general transcription factors" is as used before. The term "invasion" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. The term "transmembrane transporter" is as used before. Preferred classes of transmembrane transporters include, without limitation, proteins of the ATP-binding cassette superfamily, members of the Major Facilitator Superfamily (MFS), P-type ATPases, members of the mitochondrial carrier family (MCF) that include, without limitation, PET9; ion channels, permeases that include, without limitation, BAP2, HIP1, MEP1, and MEP2; and components that transport sugars, ions, or metals.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. The term "kinase" is as used before. Preferred kinases include, without limitation, CDC28, ELM1, FUS3, GCN2, HOG1, HSL1, HXK2, KSS1, PBS2, PHO85, RIM15, STE7, SCH9, SNF1, STE11, STE20, TPK1, TPK2, and TPK3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. The term "G-protein" is as used before. Preferred G-proteins include, without limitation CDC42, fadA, GPA1, GPA2, RAS1, and RAS2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. The term "cell surface receptor" is as used before. Preferred cell surface receptors include, without limitation, G-protein coupled receptors. Preferred G-protein coupled receptors include, without limitation, GPR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. The term "GTPase activating protein" is as used before. Preferred GTPase activating proteins include, without limitation, RGS family members. "RGS family members" are regulators of G-protein signaling that act upon G-protein coupled receptors. Preferred RGS family members include, without limitation, FlbA, RGS2, and SST2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. The term "guanine nucleotide exchange factor" is as used before. Preferred guanine nucleotide exchange factors include, without limitation, CDC24 and CDC25.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. The term "phosphatase" is as used before. Preferred phosphatases include, without limitation, CDC55 and PTC1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. The term "protease" is as used before. Preferred proteases include, without limitation, RIM13.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. The term "cyclic nucleotide phosphodiesterase" is as used

before. Preferred examples of cyclic nucleotide phosphodiesterases include, without limitation, PDE2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. The term "bacterial protein toxin" is as used before. Preferred bacterial protein toxins include, without limitation, Anthrax toxin edema factor (EF; *Bacillus anthracis*), Anthrax toxin lethal factor (LF; *Bacillus anthracis*), adenylate cyclase toxin (*Bordetella pertussis*), Cholera enterotoxin (*Vibrio cholerae*), LT toxin (*Escherichia coli*), ST toxin (*E. coli*), Shiga toxin (*Shigella dysenteriae*), Perfringens enterotoxin (*Clostridium perfringens*), Botulinum toxin (*Clostridium botulinum*), Tetanus toxin (*Clostridium tetani*), Diphtheria toxin (*Corynebacterium diphtheriae*), Exotoxin A (*Pseudomonas aeruginosa*), Exoenzyme S (*P. aeruginosa*), Pertussis toxin (*Bordetella pertussis*), alpha and epsilon toxins (*C. perfringens*), lethal toxin (LT; *Clostridium sordellii*), toxins A and B (*Clostridium dificile*), and phospholipase C (*Clostridium bifermentans*).

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on an importin protein. The term "importin" protein is as used before. Preferred examples of importin proteins include, without limitation, MSN5.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a RNA-binding protein or the product that it encodes acts on a RNA-binding protein. Preferred examples of RNA-binding proteins include, without limitation, DHH1 and WHI3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. The term "component of a SCF complex" is as used before. Preferred examples of components of a SCF complex include, without limitation, GRR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is as used before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, amyR, areA, ASH1, BAP2, BCY1, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO11, FLO8, FUS3, GCN2, GCN4, GCR1, GCR2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGS2, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes. The term "fungal homolog" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a fifth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by altering the characteristics of the fungus in a manner that is beneficial to the production of the secondary metabolite, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that alters the characteristics of the fungus. "Altering the characteristics" means changing the morphology or growth traits of the fungus. Preferred alterations include, without limitation, those alterations

that result in transition of the fungus from the hyphal to yeast form, those alterations that result in transition of the fungus from the yeast to hyphal form, alterations that lead to more or less hyphal branching, alterations that increase or decrease flocculence, adherence, cell buoyancy, surface area of the fungus, cell wall integrity and/or stability, pellet size, ability to grow at higher or lower temperatures, and alterations that increase the saturating growth density of a culture or rate of pellet formation.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, AGA1, AGA2, amyR, areA, ASH1, BAP2, BCY1, BEM1, BEM2, BEM3, BNI1, BUD2, BUD5, CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO10, FLO11, FLO5, FLO8, FLO9, FUS3, GCN2, GCN4, GCR1, GCR2, GIC1, GIC2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGA1, RGS2, RHO1, RHO2, RHO3, RHO4, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, RSR1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as

used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. The term "the gene acts on" is as used before. The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, transcription factors that modulate the expression of genes involved in the production or response to the small molecule cAMP (preferred examples include, without limitation, MGA1, MSN2, MSN4, SFL1, and SOK2); transcription factors that function downstream of mitogen-activated protein (MAP) kinase signaling pathways that regulate the yeast invasion response (preferred examples include, without limitation, MCM1, STE12, and TEC1); transcription factors that modulate the expression of genes involved in nitrogen regulation (preferred examples include, without limitation, areA, GLN3, HMS1, HMS2, nreB, tamA, and UGA3); transcription factors that modulate the

expression of genes involved in pH regulation in fungi (preferred examples include, without limitation pacC and RIM101); general transcription factors (preferred examples include, without limitation, SIN3, SNF2, SRB8, SRB9, SRB10, SRB11, SSN6, and TUP1); transcription factors that modulate the expression of genes involved in carbon metabolism (preferred examples include, without limitation, ADR1, CAT8, creA, facB, GCR1, GCR2, HAP4, MIG1, MIG2, MTH1, NRG1, OAF1, and SIP4); heme-dependent transcription factors (preferred examples include, without limitation, HAP1 and ROX1); transcription factors that modulate the expression of genes involved in the uptake of metals (preferred examples include, without limitation, AFT1, CUP9, MAC1, sreP, sreA, and ZAP1); transcription factors that modulate the expression of genes involved in cell-cycle regulation (preferred examples include, without limitation, SKN7, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in invasion (preferred examples include, without limitation, ASH1, FLO8, GTS1, INV7, MSN1, MSS11, PHD1, and RRE1); transcription factors that modulate the expression of genes involved in amino acid biosynthesis or transport (preferred examples include, without limitation, GCN4, LEU3, LYS14, MET4, MET28, MET31, metR, PUT3, sconB, and UGA3); transcription factors that modulate the expression of genes involved in phosphate metabolism or transport (preferred examples include, without limitation, PHO2 and PHO4); transcription factors that modulate the expression of genes involved in nucleotide metabolism or transport (preferred examples include, without limitation, PPR1 and UaY); transcription factors that modulate the expression of genes involved in cell wall processes (preferred examples include, without limitation, ACE2, SWI4, and SWI6); transcription factors that modulate the expression of genes involved in sporulation (preferred examples include, without limitation, IME1 and IME4); transcription factors that modulate the expression of genes involved in phospholipid synthesis (preferred examples include, without limitation, INO2); transcription factors that modulate the expression of genes involved in aflatoxin biosynthesis (preferred examples include, without limitation, aflR); transcription factors that modulate the expression of genes involved in lovastatin biosynthesis (preferred examples include, without limitation, AAD34561 and lovE); and transcription factors that modulate the expression of genes involved in filamentous fungal development (preferred examples include, without limitation, abaA). The term "general transcription factors" is as used before. The term "invasion" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. The term "transmembrane transporter" is as used before. Preferred classes of transmembrane transporters include, without limitation, proteins of the ATP-binding cassette superfamily, members of the Major Facilitator Superfamily (MFS), P-type ATPases, members of the mitochondrial carrier family (MCF) that include, without limitation, PET9; ion channels, permeases that include, without limitation, BAP2, HIP1, MEP1, and MEP2; and components that transport sugars, ions, or metals.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. The term "kinase" is as used before. Preferred kinases include, without limitation, CDC28, ELM1, FUS3, GCN2, HOG1, HSL1, HXK2, KSS1, PBS2, PHO85, RIM15, STE7, SCH9, SNF1, STE11, STE20, TPK1, TPK2, and TPK3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. The term "G-protein" is as used before. Preferred G-proteins include, without limitation CDC42, fadA, GPA1, GPA2, RAS1, RAS2, RHO1, RHO2, RHO3, RHO4, and RSR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. The term "cell surface receptor" is as used before. Preferred cell surface receptors include, without limitation, G-protein coupled receptors. Preferred G-protein coupled receptors include, without limitation, GPR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. The term "GTPase activating protein" is as used before. Preferred GTPase activating proteins include, without limitation, RGS family members. The term "RGS family members" is as used before. Preferred RGS family members include, without limitation, FlbA, RGS2, and SST2. Preferred examples of non-RGS family GTPase-activating proteins include, without limitation, BEM2, BEM3, BUD2, RGA1, and RGA2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. The term "guanine nucleotide exchange factor" is as used before. Preferred guanine nucleotide exchange factors include, without limitation, BUD5, CDC24, and CDC25.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. The term "phosphatase" is as used before. Preferred phosphatases include, without limitation, CDC55 and PTC1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. The term "protease" is as used before. Preferred proteases include, without limitation, RIM13.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. The term "cyclic nucleotide phosphodiesterase" is as used before. Preferred examples of cyclic nucleotide phosphodiesterases include, without limitation, PDE2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. The term "bacterial protein toxin" is as used before. Preferred bacterial protein toxins include, without limitation, Anthrax toxin edema factor (EF; *Bacillus anthracis*), Anthrax toxin lethal factor (LF; *Bacillus anthracis*), adenylate cyclase toxin (*Bordetella pertussis*), Cholera enterotoxin (*Vibrio cholerae*), LT toxin (*Escherichia coli*), ST toxin (*E. coli*), Shiga toxin (*Shigella dysenteriae*), Perfringens enterotoxin (*Clostridium perfringens*), Botulinum toxin (*Clostridium botulinum*), Tetanus toxin (*Clostridium tetani*), Diphtheria toxin (*Corynebacterium diphtheriae*), Exotoxin A (*Pseudomonas aeruginosa*), Exoenzyme S (*P. aeruginosa*), Pertussis toxin (*Bordetella pertussis*), alpha and epsilon toxins (*C. perfringens*), lethal toxin (LT; *Clostridium sordellii*), toxins A and B (*Clostridium dificile*), and phospholipase C (*Clostridium bifermentans*).

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on an importin protein. The term "importin" protein is as used before. Preferred examples of importin proteins include, without limitation, MSN5.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a RNA-binding protein or the product that it encodes acts on a RNA-binding protein. Preferred examples of RNA-binding proteins include, without limitation, DHH1 and WHI3.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. The term "component of a SCF complex" is as used before. Preferred examples of components of a SCF complex include, without limitation, GRR1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an adherin or the product that it encodes acts on an adherin. The term "adherin" means a molecule that functions to promote the interaction of a cell with another component, including, without limitation, interaction with other cells of the same genotype, interaction with cells of a different genotype, and interaction with growth substrates. Preferred examples of adherins include, without limitation, AGA1, AGA2, FLO1, FLO10, FLO11, FLO5, and FLO9.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA),

7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic " is as used before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34561, abaA, ACE2, ADR1, AFL1, aflR, AFT1, AGA1, AGA2, amyR, areA, ASH1, BAP2, BCY1, BEM1, BEM2, BEM3, BNI1, BUD2, BUD5,

CAT8, CDC24, CDC25, CDC28, CDC42, CDC55, CLB2, creA, CTS1, CUP9, CYR1, DFG16, DHH1, DPH3, ELM1, facB, FLO1, FLO10, FLO11, FLO5, FLO8, FLO9, FUS3, GCN2, GCN4, GCR1, GCR2, GIC1, GIC2, GLN3, GPA1, GPA2, GPR1, GRR1, GTS1, HAP1, HAP4, HIP1, HMS1, HMS2, HOG1, HSL1, HXK2, IME1, IME4, INO2, INV11, INV13, INV16, INV5, INV7, INV9, KSS1, LEU3, lovE, LYS14, MAC1, MCM1, MEP1, MEP2, MET28, MET31, MET4, metR, MGA1, MIG1, MIG2, MSN1, MSN2, MSN4, MSN5, MSS11, MTH1, NPR1, nreB, NRG1, OAF1, pacC, PBS2, PDE2, PET9, PHD1, PHO2, PHO4, PHO85, pkaR, PPR1, PTC1, PUT3, RAS1, RAS2, RGA1, RGS2, RHO1, RHO2, RHO3, RHO4, RIM101, RIM13, RIM15, RIM9, ROX1, RRE1, RSR1, SCH9, sconB, SFL1, SHO1, SHR3, SIN3, SIP4, SKN7, SNF1, SNF2, SNF7, SNF8, SOK2, SRB10, SRB11, SRB8, SRB9, sreA, sreP, SRV2, SSD1, SSN6, SST2, STE11, STE12, STE20, STE50, STE7, STP22, SWI4, SWI6, tamA, TEC1, TPK1, TPK2, TPK3, TUP1, UaY, UGA3, URE2, VPS28, VPS36, WHI3, YMR077c, YNL255c, YPR1, ZAP1, genes encoding bacterial protein toxins, and any fungal homologs of the aforementioned genes. The term "fungal homolog" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a sixth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by causing conditional lysis of the fungus, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that causes conditional lysis. "Causing conditional lysis" means causing the fungus to grow without lysis under a first set of growth conditions and to lyse under a second and different set of conditions, which are not lytic to the unmodified fungus. In preferred embodiments, the conditions that can be altered between the first and second growth conditions include, without limitation, the source or amount of nutrients such as carbon, nitrogen, and phosphate; the source or amount of specific enzymes; the source or amount of specific components found in cell walls; the amount of salts or osmolytes; the pH of the medium, the partial oxygen pressure, or temperature; and the amount of specific small molecules.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, ACE2, BCK1, BGL2, CHS1, CHS2, CHS3, CTS1, FKS1, GSC2, HOG1, ISR1, KRE6, MID2, MKK1, MKK2, PBS2, PKC1, PPH21, PPH22, PPZ1, PPZ2, PTP2, PTP3, RHO1, RLM1, ROM1, ROM2, SHO1, SKN1, SLG1, SLN1, SLT2, SMP1, SSK1, SSK2, SSK22, STE11, STT3, STT4, SWI4, SWI6, VPS45, WSC2, WSC3, WSC4, and YPD1.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor. The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, ACE2, RLM1, SMP1, SWI4, and SWI6.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. A "transmembrane transporter" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. A "kinase" is as used before. Preferred kinases include, without limitation, BCK1, HOG1, ISR1, MKK1, MKK2, PBS2, PKC1, SLT2, SSK2, and SSK22.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component involved in cell wall biosynthesis or the product that it encodes acts on a component involved in cell wall biosynthesis. Preferred classes of components involved in cell wall biosynthesis include, without limitation, glucan synthases, glucanases, chitin synthase, and chitinases. Preferred examples of components involved in cell wall biosynthesis include, without limitation, BGL2, CHS1, CHS2, CHS3, CTS1, FKS1, GSC2, KRE6, and SKN1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. A "G-protein" is a guanyl-nucleotide binding protein. Preferred G-proteins include, without limitation RHO1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. A "cell surface receptor" is as used before. Preferred cell surface receptors include, without limitation, SHO1 and SLN1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. A "GTPase activating protein" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. A "guanine nucleotide exchange factor" is as used before. Preferred guanine nucleotide exchange factors include, without limitation, ROM1 and ROM2.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. A "phosphatase" is as used before. Preferred phosphatases include, without limitation, PPH21, PPH22, PPZ1, PPZ2, PTP2, PTP3, and PTC1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. A "protease" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. A "cyclic nucleotide phosphodiesterase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. A "bacterial protein toxin" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin or the product that it encodes acts on an importin protein. An "importin" protein is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a RNA-binding protein or the product that it encodes acts on a RNA-binding protein.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. A "component of a SCF complex" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is as used before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of ACE2, BCK1, BGL2, CHS1, CHS2, CHS3, CTS1, FKS1, GSC2, HOG1, ISR1, KRE6, MID2, MKK1, MKK2, PBS2, PKC1, PPH21, PPH22, PPZ1, PPZ2, PTP2, PTP3, RHO1, RLM1, ROM1, ROM2, SHO1, SKN1, SLG1, SLN1, SLT2, SMP1, SSK1, SSK2, SSK22, STE11, STT3, STT4, SWI4, SWI6, VPS45, WSC2, WSC3, WSC4, YPD1, and fungal homologs of the aforementioned genes. The term "fungal homolog" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a seventh aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by increasing the resistance of the fungus to the deleterious effects of exposure to a secondary metabolite made by the same organism, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that increases resistance to the deleterious effects of exposure to a secondary metabolite. "Increasing the resistance of the fungus to the deleterious effects of exposure to a secondary metabolite" means to allow the fungus to survive, grow, or produce secondary metabolite in conditions that otherwise would be toxic or prevent production of secondary metabolite.

In certain embodiments of the methods according to this aspect of the invention, the modulation is overexpression of the gene. "Overexpression of the gene" is as used before. Preferred genes according to this aspect of the invention include, without limitation, AAD34558, AAD34561, AAD34564, ATR1, ERG6, ERG11, FCR1, GCN4, lovE, MDR1, PDR1, PDR3, PDR5, PDR10, PDR13, SNQ2, TRI12, YAP1, fungal homologs of the aforementioned genes, and genes that encode beta-tubulin, calcineurin (including, without limitation, CNA1), chitin

synthase, glucan synthase, HMG CoA reductase, N-terminal aminopeptidases, and RNA polymerase II.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a peptide modulator of gene expression. The term "peptide" is as used before. Peptides may be expressed in the cell or supplied exogenously. Preferably, they are provided on a scaffold to increase intracellular stability and to provide conformational constraint. Preferred peptides according to this aspect of the invention include those discussed earlier.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an activator of gene expression. The term "activator of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the peptide modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is mediated by a small molecule modulator of gene expression. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an activator of gene expression. The term "activator of gene expression" is as used before. In certain embodiments of the methods according to this aspect of the invention, the small molecule modulator is an inhibitor of gene expression. The term "inhibitor of gene expression" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transcription factor or the product that it encodes acts on a transcription factor.

The term "transcription factor" is as used before. Preferred transcription factors include, without limitation, AAD34561, FCR1, GCN4, lovE, PDR1, PDR3, and YAP1.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a transmembrane transporter or the product that it encodes acts on a transmembrane transporter. The term "transmembrane transporter" is as used before. Preferred transmembrane transporters include, without limitation, AAD34558, AAD34564, ATR1, MDR1, PDR5, PDR10, SNQ2, and TRI12.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a kinase or the product that it encodes acts on a kinase. A "kinase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a G-protein or the product that it encodes acts on a G-protein. A "G-protein" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cell surface receptor or the product that it encodes acts on a cell surface receptor. A "cell surface receptor" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a GTPase activating protein or the product that it encodes acts on a GTPase activating protein. A "GTPase activating protein" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a guanine nucleotide exchange factor or the product that it encodes acts on a guanine nucleotide exchange factor. A "guanine nucleotide exchange factor" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a phosphatase or the product that it encodes acts on a phosphatase. A "phosphatase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a protease or the product that it encodes acts on a protease. A "protease" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a cyclic nucleotide phosphodiesterase or the product that it encodes acts on a cyclic nucleotide phosphodiesterase. A "cyclic nucleotide phosphodiesterase" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a bacterial protein toxin or the product that it encodes acts on a bacterial protein toxin. A "bacterial protein toxin" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes an importin protein or the product that it encodes acts on an importin protein. An "importin" protein is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a RNA-binding protein or the product that it encodes acts on a RNA-binding protein.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a component of a SCF complex or the product that it encodes acts on a component of a SCF complex. A "component of a SCF complex" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the gene either encodes a biosynthetic enzyme or the product that it encodes acts on a biosynthetic enzyme. The term "biosynthetic enzyme" is as used before.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. The term "anti-bacterial" is as used before. Preferred anti-bacterials include, without limitation, B-lactams. Preferred B-lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- B -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- B -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is as used before. Preferred anti-hypercholesterolemics include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is as used before. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. The term "ergot alkaloid" is as used before. Preferred classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. The term "inhibitor of angiogenesis" is as used before. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. The term "glucan synthase inhibitor" is as used before. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The term "gliotoxin family of compounds" is as used before. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. The term "fungal toxin" is as used before. Preferred fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin,

griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. The term "cell surface receptor" is as used before. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. The term "plant growth regulator" is as used before. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. The term "pigment" is as defined before. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. The term "insecticide" is as used before. Preferred insecticides include, without limitation, nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. The term "anti-neoplastic" compound is as used before. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the gene is selected from the group consisting of AAD34558, AAD34561, AAD34564, ATR1, ERG6, ERG11, FCR1, GCN4, lovE, MDR1, PDR1, PDR3, PDR5, PDR10, PDR13, SNQ2, TRI12, YAP1, fungal homologs of the aforementioned genes, and genes that encode beta-tubulin, calcineurin (including, without limitation, CNA1), chitin synthase, glucan synthase, HMG CoA reductase, N-terminal aminopeptidases, and RNA polymerase II.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In an eighth aspect, the invention provides genetically modified fungi, wherein the genetically modified fungi have an ability to produce secondary metabolites and the ability of the genetically modified fungus to produce secondary metabolites has been improved by any of the methods according to the invention.

In a ninth aspect, the invention provides a method for making a secondary metabolite, the method comprising culturing a genetically modified fungus according to the invention under conditions suitable for the production of secondary metabolites. "Conditions suitable for the production of secondary metabolites" means culture conditions under which the fungus does in fact produce one or more secondary metabolite.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to limit the scope of the invention in any way.

Example 1

Preparation of clones to regulate secondary metabolite production

To prepare clones that can be used to genetically modulate the expression of genes involved in secondary metabolism, the following experiments were conducted.

The Gateway (Life Technologies, Inc.) Cloning Technology (US Patent 5,888,732) was used to generate constructs for expression of fungal regulators. The polymerase chain reaction (PCR) was used to amplify cDNA or genomic DNA containing coding sequence for fungal regulators; the resultant PCR products contain common sites at both 5' and 3' ends in order to facilitate recombination into the Gateway entry vector MB971 (see Life Technologies Inc., www.lifetech.com). The resultant entry clones were then reacted in a Gateway destination cocktail with plasmid MB1419 (or related destination vectors). MB1419 is derived from

pLXZ161. pLXZ161 is a gene vector derived from pBC-phleo (P. Silar, Fungal Genetics Newsletter 42: 73 (1995)) that carries a phleomycin resistance cassette for selection of transformants, as well as a polylinker located between the *Aspergillus nidulans PGK* promoter and the *A. nidulans trpC* terminator. pLXZ161 was constructed as follows: First, the *Aspergillus nidulans trpC* terminator was amplified from *A. nidulans* genomic DNA by PCR using Turbo Pfu Polymerase as described by the manufacturer (Stratagene,11011 North Torrey Pines Road, La Jolla, CA 92037). Primers used in this reaction are TRPC-1 5'-GCGGCCGCGGCGCCCGGCCCATGTCAACAAGAAT-3') and TRPC-2 5'-CCGCGGCCGAGTGGAGATGTGGAGT-3'. The resultant product was digested with the restriction enzymes *Sac*II and *Not*I, purified by agarose gel electrophoresis, and cloned into *Sac*II/*Not*I-digested pBC-phleo DNA, to generate pLXZ116. Second, the *A. nidulans* PGK promoter was amplified from *A. nidulans* genomic DNA by PCR using primers PGK1-1 5'-CATGGGGCCCCGTGATGTCTACCTGCCCAC-3' and PGK1-2 5'-CATGATCGATTGTGGGTAGTTAATGGTATG-3', Turbo Pfu Polymerase, and reaction conditions as described above. The resultant product was digested with *Apa*I and *Cla*I and cloned into *Apa*I/*Cla*I-digested pLXZ116, to generate pLXZ161. To produce MB1419, the *ccdB* (death gene) cassette from pEZC7201 (Life Technologies, Gateway cloning manual) was amplified by PCR using oligos MO511 (GGCCATCGATACAAGTTTGTACAAAAAAGCTGAAC) and MO512 (GCGGCCGCACCACTTTGTACAAGAAAGC), digested with *Cla*I and *Not*I, and cloned into *Not*I/*Cla*I-digested pLXZ161. This generated a destination vector in which the death gene cassette resides between the *A. nidulans PGK* promoter and the *A. nidulans trpC* terminator of pLXZ161. Thus, destination reactions using this vector allow configuration of any gene in an entry clone to be expressed under the control of the *A. nidulans PGK* promoter. The fungal selectable marker contained on this plasmid is *ble*, which confers resistance to phleomycin.

Example 2

Transformation of *Aspergillus terreus* and *Penicillium chrysogenum*

Destination clones were transformed into either *Aspergillus terreus* or *Penicillium chrysogenum*. In order to transform these fungi, spores were first generated by culture of strain

ATCC#20542 (*A. terreus*), MF1 (NRRL1951, *P. chrysogenum*), or MF20 (ATCC#11702, *P. chrysogenum*) on petri plates containing potato dextrose agar (Difco BRL) at 30°C for 3-6 days. Spores were removed from PDA either by resuspension in sterile water or Tween-80 (0.1%) or by scraping directly from the plate using a sterile spatula. Yeast extract sucrose medium, or YES (2% Yeast Extract, 6% Sucrose), was inoculated to a density of 1-5 x 10^6 spores per ml and incubated with shaking in an Erlenmeyer flask at 26-30° C for 12-16 hr (250 rpm). Mycelia were harvested by centrifugation at 3200 rpm for 10 minutes, and washed in sterile water two times. Mycelia were resuspended in a filter sterilized solution of Novozyme 234 (Sigma) at 2-5 mg/ml in 1 M $MgSO_4$ and digested at room temperature with shaking (80 rpm) for 1-2 hr. Undigested material was removed by filtration through Miracloth (Calbiochem, 10394 Pacific Center Court, San Diego, CA 92121). After adding 1-2 volumes of STC (0.8 M sorbitol, 25 mm Tris, pH 7.5, and 25 mM $CaCl_2$), the protoplasts were pelleted by centrifugation at 2500 rpm. Protoplasts were washed 2 times in STC by centrifugation. Resulting protoplasts were resuspended to a density of 5 x 10^7 per ml in a solution of STC, SPTC (40 % polyethylene glycol in STC) and DMSO in a ratio of 9:1:0.1 and frozen at –80°C. For transformations, two aliquots (100 µl each) of protoplasts were mixed with 1-5 ug of either pBCphleo or destination clones for expression of fungal regulators; mixtures were incubated on ice for 30 min. An aliquot of SPTC (15 µl) was added to each tube and the reaction was incubated at room temperature for 15 minutes. An additional aliquot (500 µl) was added with gentle mixing, and the reaction was incubated for an additional 15 minutes at room temperature. The reaction was next resuspended in 25 ml of molten regeneration medium (Potato Dextrose Agar from Sigma, 3050 Spruce Street, St Louis, MO 63103) with 0.8 M sucrose, maintained at 50° C, and poured onto a 150 mm petri plate containing 25 ml of solidified regeneration medium plus phleomycin (60-200 µg/ml for *A. terreus* and 30 µg/ml for *P. chrysogenum*). Transformants are typically visible after 2-5 days of incubation at 26–30°C.

Phleomycin resistant colonies were colony purified into small 24 well plates and then examined both on plates and in shake flask cultures. Morphological and developmental effects of the transgene were observed under both growth conditions. Due to the heterogeneous nature of transformation in filamentous fungi, at least 10 (and often many more) phleomycin resistant

colonies were pursued. Detailed examination of a subset of phleomycin resistant colonies suggests that approximately 80% of the colonies contain a transgene.

Example 3

Determination of lovastatin production

Lovastatin assays were performed using broths from shake flask cultures of *A. terreus* . *A. terreus* transformants were grown on modified RPM medium (WO/37629) containing 4% glucose, 0.3% corn steep liquor (Sigma), 0.2%KNO_3, 0.3%KH_2PO_4, 0.05%$MgSO_4.7\ H_2O$, 0.05% NaCl, 0.05% polyglycol (Dow), 0.1 % trace elements (14.3 g/l $ZnSO_4.7\ H_2O$, 2.5 g/l $CuSO_4.5\ H_2O$, 0.5 g/l $NiCl_2.6\ H_2O$, 13.8 g/l $FeSO_4.7\ H_2O$, 8.5 g/l MnSO4. H_2O, 3 g/l citric acid. H_2O (add first), 1 g/l H_3BO_3, 1 g/l Na_2MoO_4, 2.5 g/l $CoCl_2.6\ H_2O$). The final pH was adjusted to 6.5. Spores for inoculation were generated by culturing on plates containing minimal medium plus phleomycin for 1 week at 27° C. Spores for shake flask inoculation were removed from plates by dragging the tip of a sterile wooden stick approximately 1 inch across the plate surface. The tip of the stick was then dipped into the shake flask medium and swirled gently. Cultures were grown at 27° C, 225 RPM for 5-6 days.

Quantitative assays were performed to assess the levels of lovastatin in broths from shake flask cultures. To assay lovastatin production, $(His)_6$HMGCoA reductase was first expressed in *Saccharomyces cerevisiae* and purified with a nickel column. *A. terreus* samples were fermented as described above and 0.5 mL samples were taken at day 5-6, put in a 1 mL 96-well plate, and centrifuged to remove mycelia before assaying. Samples were transferred to another 1 mL 96-well plate and frozen at –80°C.

Samples were thawed and 10 µL removed and diluted 1:50 in H_2O. 10 µL of this diluted broth was assayed in a reaction (200 µL total) containing 1 mM L-HMGCoA, 1 mM NADPH, 0.005 mM DTT and 5 µL $(His)_6$HMGCoA reductase. The disappearance of absorbance at 340 nm was observed over time, and this represents the utilization of NADPH, an electron donor required for the reduction of HMGCoA. Lovastatin inhibits HMGCoA reductase, and thus assays containing lovastatin display a decreased rate of disappearance of absorbance at 340 nm. The initial velocities for NADPH disappearance were calculated for broth-containing samples

and reactions containing lovastatin standards. Velocities were then adjusted for dilution, and regression analysis was used to determine metabolite concentration.

Several fungal regulators were found to improve the overall yield of lovastatin in shake flask cultures. It is possible that these regulators will also increase productivity. Lovastatin production levels from strains containing regulators were compared to either levels from strains containing control vector or a non-transformed strain. Data points were collected for at least 10 phleomycin resistant colonies, and the production levels for each sample set was displayed as a box plot (*e.g.*, Figure 3). In box plot portrayals of the data, the box represents the central 50% of the data, and the line within the box represents the median value for the entire data set; outlying data points are flagged. Box plot portrayals assist in determining whether a particular sample set is significantly different from a set collected from a control strain.

Table 1 displays representative fungal regulators that improved the yield of lovastatin in shake flask cultures.

Table 1.

Plasmid Name	Regulator
MB1423	pacC (DNA-binding domain (DBD))-VP16 (transcription activation domain (TAD))
MB1695	VP16 (TAD)-pacC (DBD)
MB1564	VP16 (TAD)-pacCL266
MB2415	amdAG229D (TAD)-pacCL266
MB2417	amdAG229C (TAD)-pacCL266
MB2418	amdAG229D (TAD)-pacC (DBD)
MB2419	amdAG229C (TAD)-pacC (DBD)
MB2203	VP16 (TAD)-An09
MB1316	lovE
MB2244	VP16 (TAD)-Pc23
MB1970	At18
MB1310	creA

Box plots are displayed in Figure 3. Hutchinson *et al.*, PCT Publication WO 00/37629, has demonstrated that overexpression of lovE increases lovastatin production in *Aspergillus terreus*; thus, lovE expressing strains served as positive controls in these experiments. The data in Figure 3 is organized in sets of three; samples expressing a particular regulator are always compared to control samples (both positive and negative) grown and assayed at the same time.

The results in Figure 3 indicate that several fungal regulators appreciably stimulate production of lovastatin.

Example 4

Determination of penicillin production

Penicillin assays were performed using broths from shake flask cultures of *P. chrysogenum*. To test levels of penicillin produced in *P. chrysogenum* transformants, a plug containing spores and mycelia was used as the inoculum. The published P2 production medium (J Lein (1986) in Overproduction of microbial metabolites (Z. Vanek and Z. Hostalek eds.) pp. 105-139), which contains, 30% lactose, 5X pharmamedia cotton seed flour, ammonium sulfate, calcium carbonate, potassium phosphate, potassium sulfate, and phenoxyacetic acid pH 7, was used. Flasks were incubated at 26° C with shaking at 225 rpm, and sampling was done after 6 days of growth.

To monitor penicillin production, 1-1.5 mls of broth was placed into 96-well plates. The fermentation broth was clarified by centrifugation for 10 min at 4000 g. Supernatants were transferred to a new 96-well plate. Standard samples containing 0, 25, 50, 100, 200, 300, 400, 500 μg/mL phenoxymethylpenicillin (sodium salt) were dissolved in 10 mM potassium phosphate (pH 7.0). For penicillin assays 40 μL of clarified fermentation broth and penicillin standard solutions were transferred to a 96-well UV, collection plate. 200 μL of imidazole reagent was placed in a 96-well filter plate (0.45 micron). The imidazole reagent was prepared by dissolving 8.25 g of imidazole in 60 mL of water, adding 10 mL of 5 M HCl and then adding 10 mL of mercuric chloride solution (0.27 g dissolved in 100 mL of water). The pH of the imidazole reagent was adjusted to 6.80 +/- 0.05 with 5 M HCl and then diluted to 100 mL with water (see *e.g.*, Bundgaard, H. and K. Ilver, Journal of Pharm Pharmac 24: 790-794 (1972)). The derivatization reaction of penicillin was initiated by vacuum filtration of imidazole reagent into a collection plate containing the aliquoted samples and standards. The collection plate was placed into the 96-well plate reader at 45°C, and an increase at 325 nm was monitored over 20 minutes. A Molecular Devices 96-well UV/Vis plate reader was used for all spectrophotometric detection.

Several fungal regulators were found to improve the yield of penicillin in shake flask cultures. These experiments were performed in both MF1 (NRRL1951), an early strain in the

penicillin development series, and MF20 (ATCC#11702), a strain of *Penicillium chrysogenum* that produces approximately ten-fold more penicillin than MF1. As described above for lovastatin, large numbers of phleomycin resistant colonies were used in shake flask experiments, such that analysis could be performed to determine whether the effect of a particular regulator was statistically significant. Strains of MF20 expressing pacCL266 (MB1563), an alkalinity mimicking allele of pacC, displayed increased penicillin production. pacC (DBD)-VP16 (TAD) (*e.g.*, MB1423) stimulated penicillin production in MF1. In addition, both shake flask and small-scale bioreactor studies demonstrate that this regulator can improve the productivity of Penicillium strains; strains expressing pacC (DBD)-VP16 (TAD) initiate production and reach maximum production levels earlier than the parent MF1 strain or a strain transformed with a control vector. Regulators from fungi other than *Penicillium chrysogenum* also were found to improve penicillin production. Both MF1 and MF20 strains that expressed *lovU* (MB1317), a gene from *Aspergillus terreus*, displayed increased yields of penicillin production. Penicillin yields were also improved in MF20 strains that expressed *YHR056c*, a gene from *Saccharomyces cerevisiae*.

These results demonstrate that many fungal regulator genes are capable of improving penicillin productions, including genes from unrelated species.

Example 5

Alteration of fungal morphology

In addition to improving yield or productivity, several other traits can be modulated in order to improve the process of production of secondary metabolites in fungi. Desired traits would include altering morphological characteristics that would be favorable to a particular fermentation. Several fungal regulators were found to alter morphological or developmental characteristics of *Penicillium* strains. Specifically, pacC (DBD)-VP16 (TAD) (*e.g.*, MB1423) and VP16 (TAD)-areA (from *Penicillium chrysogenum)* (MB2220) caused hyphae to aggregate in shake flask cultures. Pellet size is often a critical factor during growth in bioreactors. Pellet size can impact variables during growth such as the amount of energy needed to drive the impellers within the bioreactor. Aggregating cultures also can be beneficial for purification of biomass from culture broth during post-fermentation processing. In addition to these

morphological effects, expression of pacC (DBD)-VP16 (TAD) (*e.g.*, MB1423), VP16 (TAD)-areA (MB2220), At32 (from *Aspergillus terreus*) (MB1623), and VP16 (TAD)-At32 (MB2200) affected the developmental process of sporulation. Strains expressing pacC (DBD)-VP16 (TAD), VP16 (TAD)-areA, and VP16 (TAD)-At32 are sporulation defective, whereas strains expressing At32 sporulate in submerged culture. In some instances (*e.g.*, sterigmatocystin biosynthesis in *Aspergillus nidulans*) sporulation and production of secondary metabolites are coordinately regulated. In other examples, such as penicillin production, sporulation defective strains often produced increased levels of metabolite. Therefore, regulators that increase or decrease sporulation may provide a tool to adjust the developmental state of the fungus to the optimal state for production of any particular metabolite.

Example 6

Reducing toxic effects of a secondary metabolite

Other desired traits would include increasing resistance to the deleterious effects of exposure to a secondary metabolite,

Growth of a fungus that produces secondary metabolites can be limited, in part, by the toxic effects of the secondary metabolites themselves. In the absence of resistance mechanisms to protect fungi from toxic effects of these metabolites, decreased yields of the metabolite can be observed. For example, Alexander *et al.* (Mol. Gen. Genet. 261: 977-84 (1999)) have shown that the trichothecene efflux pump of *Fusarium sporotrichiodes*, encoded by the gene *TRI12*, is required both for high level production of, and resistance to the toxic effects of, trichothecenes produced by this fungus. Thus, modifications that increase the resistance of a fungus to a toxic secondary metabolite that it produces can increase the saturation density and extend the metabolically active lifetime of the producing fungus. In a bioreactor, such attributes will have the beneficial effect of increasing yield and productivity of a metabolite. Regulators of secondary metabolite production whose expression can be modulated to increase resistance of a fungus to toxic metabolites that it produces can include, without limitation, transporters that promote efflux of the metabolite from cells, enzymes that alter the chemical structure of the metabolite within

cells to render it non-toxic, target(s) of the metabolite that mediate its toxicity, and gene products that alter cellular processes to counteract the toxic effects of a metabolite. Additional benefits of increasing efflux of secondary metabolites include increasing the amount of metabolite available for purification from the fermentation broth and mitigation of feedback inhibition of secondary metabolism that may be mediated by the metabolite itself. Indeed, feedback inhibition of a biosynthetic pathway by a product of that pathway is well documented in many microorganisms, and this inhibition can act at the transcriptional, translational, and post-translational levels. Several well-documented examples in yeast include the transcriptional repression of lysine biosynthetic genes by lysine (Feller *et al.*, Eur. J. Biochem. 261: 163-70 (1999)), the decreased stability of both the mRNA encoding the uracil permease Fur4p and the permease itself in the presence of uracil (Seron *et* al., J. Bacteriol. 181: 1793-800 (1999)), and the inhibition of alpha-isopropyl malate synthase, a key step in leucine biosynthesis, by the presence of leucine (Beltzer *et al.*, J. Biol. Chem. 263: 368-74 (1988)).

Transporters that could mediate resistance to secondary metabolites include members of the major facilitator superfamily (MFS) and the ATP binding cassette (ABC) transporters. For example, overexpression of the class I MFS-type transporter Flr1p in *S. cerevisiae* has been shown to confer resistance to a variety of toxic compounds such as cycloheximide, fluconazole, 4-nitroquinolone oxide, and cerulenin (Alarco *et* al., J. Biol. Chem. 272: 19304-13 (1997); Oskouian and Saba, Mol. Gen. Genet. 261: 346-53 (1999)). MFS transporters have been functionally grouped into 23 families in yeast, several of which contain members known or suspected to mediate resistance to toxic compounds by promoting their efflux from the cell (reviewed by Nelissen *et al.* in FEMS Microbiol. Rev. 21: 113-34 (1997)). Likewise, ABC transporters encoded by genes including *PDR5* from *S. cerevisiae* (Boyum and Guidotti, Biochem. Biophys. Res. Commun. 230: 22-6 (1997)), *PMR1* from *Penicillium digitatum* (Nakuane *et al.*, Appl. Environ. Microbiol. 64: 3983-8 (1998)) and *MDR1* from *Candida albicans* (Sanglard *et al.*, Antimicrob. Agents Chemother. 39: 2378-86 (1995)), amongst others, have been shown to confer resistance to a variety of toxic compounds when their expression is increased. A complete cataloging of ABC transporters in yeast, as well as predicted function based on sequence similarities to transporters of known function, is described in (Decottignies and Goffeau, Nat. Genet. 15: 137-45 (1997)).

Transcription factors that regulate the expression of efflux pumps could also be used to increase efflux of a drug from a fungal cell to increase yields of a metabolite and decrease toxicity of the secondary metabolite in a fermentation. Such transcription factors include, but are not limited to, genes such as *YAP1*, *PDR1*, and *PDR3* from *S. cerevisiae* and their homologs. Overexpression of each of these genes has been shown to upregulate expression of transporters and cause increased resistance of *S. cerevisiae* to toxic compounds (for examples, see Reid *et al.*, J. Biol. Chem. 272: 12091-9 (1997); Katzmann *et al.*, Mol. Cell. Biol. 14: 4653-61 (1994); Wendler *et al.*, J. Biol. Chem. 272: 27091-8 (1997)).

Resistance to the toxic effects of secondary metabolites mediated through modulating expression of target genes will vary with metabolite. For example, amatoxins kill cells by inhibiting the function of the major cellular RNA polymerase, RNA polymerase II, in eucaryotic cells. Mutant forms of RNA polymerase II resistant to the effects of alpha-amanitin have been described (Bartolomei *et al.*, Mol. Cell. Biol. 8: 330-9 (1988); Chen *et al.*, Mol. Cell. Biol. 13: 4214-22 (1993)). Similarly, mutations affecting HMG CoA reductase, the target enzyme for the secondary metabolite lovastatin, have been identified. Increased levels of HMG CoA Reductase can also cause resistance to lovastatin (Ravid *et al.*, J. Biol. Chem. 274: 29341-51 (1999); Lum *et al.*, Yeast 12: 1107-24 (1996)). Taxol (paclitaxel), causes lethality by increasing microtubule stability, thus preventing exit from mitosis. Dominant mutations affecting beta-tubulin that confer resistance to taxol have been characterized (for example, see Gonzalez *et al.*, J. Biol. Chem. 274: 23875-82 (1999)) and could prove to be useful to confer resistance of production strains to this toxic metabolite. Such mutations appear to decrease the stability of microtubules; whether these mutations affect the binding of taxol to microtubules is not known. Similarly, modulating expression of other genes that decrease the stability of microtubules could also confer taxol resistance to a fungus that produces taxol. The pneumocandin and echinocandin families of metabolites are fungal secondary metabolites that inhibit the enzyme 1,3-beta-D-glucan synthase. Dominant mutations in the *Candida albicans* glucan synthase gene, *FKS1*, have been shown to confer resistance to candins (Douglas *et al.*, Antimicrob. Agents Chemother. 41: 2471-9 (1997)). Glucan synthase mutations such as these could be used to generate fungal production strains with increased resistance to the candin class of antifungals. *S. cerevisiae* mutants resistant to the growth-inhibitory effects of the fungal secondary metabolite cyclosporin A have also been

described (Cardenas *et al.*, EMBO J 14: 2772-83 (1995)). These mutants were shown to harbor mutations in *CNA1*, the gene encoding the catalytic subunit of the heterodimeric calcium-calmodulin dependent phosphatase, calcineurin A. Fumagillin, an antiangiogenic agent, binds to and inhibits the N-terminal aminopeptidases in a wide variety of both procaryotes and eucaryotes (Sin *et al.*, Proc. Natl. Acad. Sci. USA 94: 6099-103 (1997), Lowther *et al.*, Proc. Natl. Acad. Sci. USA 95: 12153-7 (1998)). Mutations in this enzyme that block fumagillin binding and/or inhibitory activity could well prove useful in enhancing the resistance of fungal production strains to the growth inhibitory effects of this secondary metabolite.

To demonstrate the feasibility of engineering a fungal strain to be resistant to otherwise toxic amounts of a secondary metabolite, two genes from the lovastatin biosynthetic cluster of *A. terreus* strain ATCC 20542 were used (Kennedy *et al.*, Science. 284: 1368-72 (1999)). These genes are predicted to encode proteins, denoted by Genbank accession numbers AAD34558 (hereafter referred to as PUMP1) and AAD34564 (hereby referred to as PUMP2), that are members of the MFS class of transporters. As described above, some MFS transporters are known to confer resistance to toxic compounds. PUMP1 and PUMP2 were tested for their ability to confer resistance to otherwise toxic levels of lovastatin when expressed in the fungus *S. cerevisiae.*

Aspergillus terreus (MF22; ATCC#20542) was grown for 45 hours in Production Media at 25°C (Production Media contains Cerelose, 4.5% (w/v) Peptonized Milk, 2.5% (w/v) Autolyzed yeast, 0.25% (w/v) Polyglycol P2000, 0.25% (w/v) pH to 7.0). Mycelia were harvested in a 50cc syringe plugged with sterile cotton wool using a vacuum apparatus, washed once with sterile H2O, and snap frozen in liquid nitrogen. Mycelia were then ground to a powder under liquid nitrogen in a mortar and pestle, and homogenized in RLC buffer (Qiagen RNeasy Kit; Qiagen Inc., 28159 Avenue Stanford, Valencia CA 93155) using a GLH rotor-stator homogenizer (Omni International, 6530 Commerce Ct., Suite 100, Warrenton, VA 20817.) Total RNA was purified using a RNeasy Maxi column according to the instructions of the manufacturer.

The polyA+ fraction of the *A. terreus* total RNA was isolated using Oligotex beads (Qiagen Inc.). Purified polyA+ RNA (5 μg) was used to generate complementary DNA (cDNA)

using Superscript Reverse Transcriptase (Gibco BRL, 9800 Medical Center Drive, PO Box 6482, Rockville, MD 20849) according to the instructions of the manufacturer.

The cDNA was then used to isolate and clone PUMP1 and PUMP2 gene sequences using the polymerase chain reaction (PCR) and Gateway (Life Technologies) Cloning Technology (US Patent 5,888,732). Oligonucleotide sequences used for PCR were 5'-ACAAAAAAGCAGGCTCCACAATGACATCCCACCACGGTGA-3' (SEQ ID NO: 7) and 5'-ACAAGAAAGCTGGGTTCATTCGCTCCGTCCTTTCT-3' (SEQ ID NO: 8) for PUMP1. Oligonucleotide sequences used for PUMP2 PCR were 5'-ACAAAAAAGCAGGCTCCACAATGGGCCGCGGTGACACTGA-3' (SEQ ID NO: 9) and 5'-ACAAGAAAGCTGGGTCTATTGGGTAGGCAGGTTGA-3' (SEQ ID NO: 10). The resultant plasmids, MB1333 and MB1334, were designed to express PUMP1 and PUMP2, respectively, under control of the *S. cerevisiae* promoter TEF1. The plasmids carry a functional *URA3* gene to allow for selection of the plasmid on media lacking uracil in a *ura3* mutant strain. These plasmids also contained a 2-micron origin for high-copy replication in yeast. Control plasmids were as follows: MB969, the parent vector for MB1333 and MB1334, that does not contain a heterologous gene and is not expected to confer resistance to a yeast strain; MB1344, constructed and described in Donald *et al.*, Appl. Environ. Microbiol. 63: 3341-4 (1997) as pRH127-3, that expresses a soluble form of HMG CoA reductase under control of the yeast *GPD1* promoter and is known to confer resistance to increased levels of lovastatin (Donald *et al.*, Appl. Environ. Microbiol. 63: 3341-4 (1997)).

MB1333, MB1334, MB969 and MB1344 were transformed into the yeast strain 22409 (Research Genetics, USA) using standard transformation methods for *S. cerevisiae* (Biotechniques, 1992, 13(1): 18). Strain 22409 is derived from the S288c strain background of *S. cerevisiae*, and its complete genotype is as follows: *MATa/α, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, ura3Δ0/ura3Δ0, LYS2/lys2Δ0, MET15/met15Δ0 pdr5::G418/PDR5.* Transformants were grown overnight at 30°C in synthetic complete media lacking uracil (SC-U) to maintain selection for the plasmid. Cultures were diluted 1:10 in sterile water, and 5µl of each strain was spotted to SC-URA agar containing different concentrations of lovastatin as shown in Figure 1. Strikingly, the strain harboring MB1333, and thus expressing PUMP1, shows resistance to lovastatin equivalent to the positive control strain in which the soluble fragment of HMG CoA reductase is

overexpressed (strain carrying MB1344). These strains show no obvious growth inhibition even at the highest concentrations of lovastatin tested (150μg/ml). In contrast, the vector-only control and the strain expressing PUMP2 show growth inhibition at the lowest concentration of lovastatin tested (50 μg/mL). Thus, these data indicate that PUMP1 is an excellent candidate for use in engineering lovastatin producing strains to enhance resistance to lovastatin and to promote efflux of this secondary metabolite.

Example 6

Causing conditional lysis of a fungus

Methods for improving the production of secondary metabolites can involve the construction of strains with desired characteristics for growth or recovery of secondary metabolites. Optimal strain characteristics likely will vary depending upon the fungus being utilized, the particular secondary metabolite being produced, and the specifications of an individual fermentation apparatus. Two traits that might be advantageous for maximal production of secondary metabolites are strains that can be lysed under specific conditions and strains that have morphological characteristics such as increased surface area of active growth and decreased hyphal length. Described below are examples of how both of these traits can be affected by modulating the activity of small GTP-binding proteins (G-proteins).

Fungi must respond to adverse external signals such as osmotic stress. Media for production of secondary metabolites often are hypo-osmotic, whereas fungi that exist on desiccated surfaces must respond to hyper-osmotic stress. One response to hyper-osmotic conditions is to increase the intracellular concentration of osmolytes such as glycerol. During hypo-osmotic stress the integrity of a fungal cell can be maintained both by decreasing intracellular osmolyte concentrations as well as by cell wall modifications. In *Saccharomyces cerevisiae* the *PKC1-SLT2* signaling pathway is required for growth in conditions of low osmolarity (reviewed in Heinisch *et al.*, Mol. Microbiol. 32: 671-680 (1999)). *PKC1*, which encodes yeast protein kinase C, is activated by components such as the small GTP-binding protein Rho1. Pkc1 then transduces this signal to a MAP kinase signaling cascade that includes

the MEK kinase Bck1, the functionally redundant MEKs Mkk1 and Mkk2, and the MAP kinase Slt2. Mutations in genes encoding these signaling components result in varying degrees of cell lysis on media of low osmolarity. Genetic screens have identified many other proteins that function either upstream of *PKC1-SLT2* signaling or regulate specific pathway components. These factors include Ppz1, Ppz2, Pph21, Pph22, Ptp2, Ptp3, Isr1, Rom1, Rom2, Mid2, Slg1, Wsc2, Wsc3, Wsc4, Stt3, Stt4, and Vps45; many of these components have homologs in other fungi. In addition, transcription factors, such as Rlm1, Swi4, and Swi6, that can function downstream of *PKC1-SLT2* signaling have been identified, and it has been demonstrated that some of these factors are required for the proper expression of genes involved in cell wall biosynthesis. Thus, many components that can modulate the structural integrity of yeast cells have been identified. It is possible that manipulation of these factors could be performed, such that conditional expression of variants of these genes (or the homologs from filamentous fungi) would result in the lysis of fungi and maximal recovery of secondary metabolites.

Conditional lysis of fungi at the conclusion of a fermentor run would be a powerful method for promoting increased recovery of secondary metabolite. Preferably, conditional lysis would require a simple manipulation such as a change in a standard growth parameter (*e.g.* temperature, dissolved oxygen) or addition of an inexpensive solute. Examples of small molecules that may cause cell lysis include the protein kinase C inhibitor staurosporine, caffeine, dyes that bind the cell wall polymer chitin (*e.g.* calcofluor white, Congo red), inhibitors of glucan synthase (*e.g.* candins), and inhibitors of chitin synthase. The cost of using these molecules in a large-scale fermentor likely would be prohibitive. Similarly, addition of enzymes such as glucanases or chitinases would likely be an effective, but costly, method for inducing lysis. An alternative means to induce lysis would be the conditional expression of a dominant negative mutation in a gene encoding a component required for cell wall integrity. Since many components of the *PKC1-SLT2* signaling pathway are widely conserved, it is possible that the conditional expression of a dominant inhibitory form of a member of this pathway would facilitate lysis in a variety of fungi, including those fungi that produce secondary metabolites such as lovastatin and cyclosporin A.

The G-protein Rho1 functions to regulate cell wall integrity by at least two independent mechanisms; Rho1 activates Pkc1 signaling as well as 1,3-beta-glucan synthase activity (Nonaka

et al., EMBO J. 14: 5931-5938 (1995); Drgonova *et al.*, Science 272: 277-279 (1996); Qadota *et al.*, Science 272: 279-281 (1996)). In addition, dominant inhibitory forms of Rho1 have been identified. Expression of a *rho1G22S D125N* mutant form in a wild-type *Saccharomyces cerevisiae* strain results in cell lysis. Therefore, the conditional expression of dominant inhibitory forms of Rho1 under the control of a heat-shock inducible promoter might be an effective method for causing cell lysis in production fungi.

RHO1 coding sequence for construction of dominant mutations can be isolated from *Saccharomyces cerevisiae* genomic DNA. Primers 5'-cgc**GGATCC**CGACATATTCGAGGTTGACT-3' (SEQ ID NO: 11) and 5'-ccc**AAGCTT**GCTAGAAATATGAACCTTCC-3' (SEQ ID NO: 12) are used to amplify *RHO1* coding sequence with 1 kilobase of upstream regulatory sequence and 500 basepairs of downstream regulatory sequence. *Bam*HI and *Hind*III restriction sites are added to the oligonucleotides to facilitate cloning into the pRS416 centromere-based yeast vector. The Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla CA) is used to first create a mutation that encodes the G22S substitution; next, the pRS416*rho1G22S* plasmid is used as a template to introduce a mutation that encodes the D125N substitution. Primer pair 5'-gtgcctgtAgtaagacatgt-3' / 5'-acatgtcttacTacaggcac-3' is used to anneal to the pRS416*RHO1* template for pRS416*rho1G22S* allele construction. Primer pair 5'-gtaaagtgAatttgagaaac-3' / 5'-gtttctcaaatTcactttac-3' is used to anneal to the pRS416*rho1G22S* template for pRS416*rho1G22S D125N* allele construction. pRS416*rho1G22S D125N* and control plasmids (pRS416*RHO1* and pRS416) are then used to transform a wild-type *ura3* auxotrophic strain. Transformants are selected and grown at 25°C in synthetic liquid growth medium lacking uracil and containing the osmolyte sorbitol (1M). Cultures are then transferred to growth in synthetic liquid growth medium lacking uracil without sorbitol, and cells are visually inspected following growth for various periods of time. Expression of the *rho1G22S D125N* dominant allele causes cell lysis after growth for approximately 120 minutes.

Conditional promoters can be used to express *RHO1* dominant mutations in filamentous fungi. The *Aspergillus niger tpsB* gene is expressed at low levels during growth at ambient temperatures, whereas expression is strongly enhanced upon heat-shock at 40°C; *tpsB* regulatory sequence contains multiple copies of the CCCCT stress responsive element (Wolschek *et al.*, J.

Biol. Chem. 272: 2729-2735 (1997)). Primers 5'-catg**GGGCCC**TCTCTCCACCGGCACTAAGATAGC-3' (SEQ ID NO: 13) and 5'-cgc**GGATCC**ag**CAT**TGGAAAAGGAGGGGGGGGAAG-3' (SEQ ID NO: 14) are used to amplify 490 basepairs of *tpsB* upstream regulatory sequence from *A. niger* genomic DNA. This PCR product contains the *tpsB* start codon followed by a *Bam*HI cloning site. The *tpsB* upstream regulatory sequence can be cloned as an *ApaI/Bam*HI fragment into the filamentous fungal vector pLXZ116 (see Example 1). The *tpsB* promoter is cloned into a multiple cloning site that also contains terminator sequence of the *A. nidulans trpC* gene. Primers 5'-cgc**GGATCC**aTCACAACAAGTTGGTAACAGTATC-3' (SEQ ID NO: 15) and 5'-gg**ACTAGT**TAACAAGACACACTTCTTCTTCTT-3' (SEQ ID NO: 16) are used to amplify *rho1G22S D125N* coding sequence, and the product is cloned into the *Bam*HI/*Spe*I sites of the *tpsB* containing filamentous fungal vector. This vector can be used to conditionally express (at 40°C) a dominant negative form of Rho1 that can cause cell lysis.

The filamentous fungal vector containing the *tpsB* promoter (no *RHO1* insert) and a vector containing *rho1G22S D125N* are used to transform *Aspergillus nidulans*, *Penicillium chrysogenum*, and *Aspergillus terreus*. To assess the impact of conditional expression of a *RHO1* dominant negative mutation on cell wall integrity of filamentous fungi, mycelia or spore preps are made from 10 independent PCR-positive transformants, and mycelia or spores are used to inoculate both liquid shake flask cultures and plates containing minimal or rich medium. After growth for 1-2 days the strains are transferred to both 37°C and 40°C. Strains are examined for morphological defects over the next 24 hours of incubation; potential morphological defects include abnormalities in polarized growth, hyphal wall integrity, and conidiophore development. The optimal time of heat-shock induction required for lysis will be determined. Furthermore, it will be determined whether any abnormalities can be suppressed by growth on medium containing osmotic stabilizers such as sorbitol (1.2 M), sucrose (1 M), or NaCl (1.5 M).

Transformants of *Aspergillus terreus* that display morphological abnormalities are used to assess whether conditional lysis of strains can be a tool for recovering larger quantities of lovastatin from fermentation broths. Five independent PCR-positive *RHO1*-containing transformants that display lysis defects will be processed as the *A. terreus* transformants

described in earlier examples. Cultures from each transformant and control strains will be grown for either 8, 9, 10, 11, or 12 days, and cultures will then be incubated at the optimal temperature and for the optimal time required for cell lysis. Following heat shock the cell mass from each culture is separated from the broth by filtration, and the cell mass is lyophilized and weighed. Lovastatin concentration in the broth is calculated as described in earlier examples.

Morphological characteristics such as decreased hyphal length might be advantageous during production of secondary metabolites. For example, strains with shorter filament lengths should display decreased entanglement, floc formation, and shear stress. Such strains would be less susceptible to shear stress damage, these strains might reduce viscosity and facilitate mass transfer, and short filament strains might save energy costs required to power impellers. Increasing the amount of hyphal branching should result in an overall decrease in filament length. The following example describes how expression of a dominant inactive form of the *Saccharomyces cerevisiae* Rsr1 protein (also known as Bud1) results in increased lateral branch formation.

The yeast Rsr1 protein is required for proper bud site selection; strains lacking Rsr1 bud at random sites on the cell surface. Dominant negative mutations such as *rsr1K16N* have been identified, and expression of these mutant forms cause random bud site selection without causing obvious growth defects. Expression of *rsr1K16N* in filamentous fungi may increase branching, decrease filament length, and not have deleterious effects on the growth of the organism.

RSR1 coding sequence for construction of dominant mutations can be isolated from *Saccharomyces cerevisiae* genomic DNA. Primers 5'-cgc**GGATCC**TATCTTCACTCAATATACTTCCTA-3' (SEQ ID NO: 17) and 5'-ccc**AAGCTT**CATCGTTGAAACTTGATAACGCAC-3' (SEQ ID NO: 18) are used to amplify *RHO1* coding sequence with 750 basepairs of upstream regulatory sequence and 500 basepairs of downstream regulatory sequence. *Bam*HI and *Hind*III restriction sites are added to the oligonucleotides to facilitate cloning into the pRS416 centromere-based yeast vector. The Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla CA) is used to create dominant-negative *RSR1* substitution mutation K16N. Primer pairs 5'- tggtgtcggtaa**T**tcctgcttaac-3' / 5'-gttaagcagga**A**ttaccgacacca-3' is used to anneal to the pRS416*RSR1* template for allele construction. The pRS416*rsr1K16N* and control pRS416 plasmids are then used to transform a

haploid wild-type *ura3* auxotrophic strain. Transformants are selected and grown at 30°C in YPD liquid growth medium. Log phase cultures are fixed in 3.7% formaldehyde (vol:vol) and stained with the chitin-binding dye Calcofluor white, as described; previous sites of bud formation are marked with a chitin-rich structure called a bud scar. Fluorescent microscopy reveals that cells containing the control plasmid display clustering of bud scars at one pole of the cells, the well-characterized haploid pattern of bud site selection. Cells expressing *rsr1K16N* display a random pattern of bud site selection; bud scars are scattered across the surface of haploid cells. Cells expressing *rsr1K16N* do not display other obvious growth or morphological defects.

The *Aspergillus nidulans* PGK promoter can be used to express *RSR1* dominant mutations in filamentous fungi. A filamentous fungal vector containing a multiple cloning site that is flanked by the PGK promoter and terminator sequence of the *A. nidulans trpC* gene is used. Primers 5'-cgc**GGATCC**GACTAATGAGAGACTATAAATTAG-3' (SEQ ID NO: 19) and 5'-ccg**CTCGAG**CTATAGAATAGTGCAAGTGGAAGC-3' (SEQ ID NO: 20) are used to amplify *rsr1K16N* coding sequence, and the product is cloned into the *Bam*HI/*Xho*I sites of the filamentous fungal vector. This vector can be used to express a dominant negative form of Rsr1 that will affect the process of selecting sites for polarized growth.

The filamentous fungal vector containing *rsr1K16N* and control vector are used to transform *Aspergillus nidulans*, *Penicillium chrysogenum*, and *Aspergillus terreus*. To assess the impact of expression of *RSR1* dominant negative mutations on lateral branch formation and filament length, mycelia and spore preps are made from 10 independent PCR-positive transformants, and mycelia and spores are used to inoculate both liquid shake flask cultures and plates containing minimal or rich medium. Strains are examined at various timepoints over a 48 hour period for morphological alterations, including altered patterns of germ tube emergence, increased lateral branching, decreased filament length, alterations in hyphal width, and changes in chitin staining pattern. Strains displaying desirable morphological changes are then tested in shake flask conditions to determine whether levels of penicillin (*A. nidulans*, *P. chrysogenum*) or lovastatin (*A. terreus*) production have changed significantly.

Aspergillus terreus and *Penicillium chrysogenum* transformants that display morphological characteristics such as decreased filament length and produce expected or greater

levels of lovastatin and penicillin, respectively, are used to assess whether morphological changes can impact upon bioreactor challenges such as shear stress damage, mass transfer, and energy costs. Five independent PCR-positive *RSR1*-containing transformants that display morphological alterations are grown in a small-scale bioreactor, and examined for improved fermentation characteristics and/or production of secondary metabolite.

What is claimed is:

1. A method for improving production of a secondary metabolite by a fungus by increasing the yield of the secondary metabolite in the fungus, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that improves the yield of the secondary metabolite, provided however, that when the secondary metabolite is isopenicillin N, then the modulation is not mediated by transcription factor CPCR1, and when the gene involved in regulation of secondary metabolite production is from *Saccharomyces cerevisiae*, then the modulation is not through decreased activity or expression of HOG1, BEM2, RIM15, SFL1, IRA1, SSD1, SRB11, SWI4, TPK3 or though increased activity or expression of AFL1, DHH1, INV7, INV8, STE21, PET9, MEP2, INV1, INV5, INV6, INV9, INV10, INV11, INV12, INV13, INV14, INV15, CDC25, MCM1, MGA1, PHD2, PHO23, PTC1, RIM1, STP22, TPK2 or YPR1.

2. The method according to claim 1, wherein the modulation is overexpression of the gene.

3. The method according to claim 1, wherein the modulation is conditional expression of the gene.

4. The method according to claim 1, wherein the modulation is expression of a dominant mutation of the gene.

5. The method according to claim 4, wherein the dominant mutation is a dominant negative mutation.

6. The method according to claim 4, wherein the dominant mutation is a dominant positive mutation.

7. The method according to claim 4, wherein the dominant mutation is a dominant neomorphic mutation.

8. The method according to claim 1, wherein the modulation is mediated by a transcription factor.

9. The method according to claim 1, wherein the modulation is mediated by a peptide modulator of gene expression.

10. The method according to claim 9, wherein the peptide modulator is an activator of gene expression.

11. The method according to claim 9, wherein the peptide modulator is an inhibitor of gene expression.

12. The method according to claim 1, wherein the modulation is mediated by a small molecule modulator of gene expression.

13. The method according to claim 12, wherein the small molecule modulator is an activator of gene expression.

14. The method according to claim 12, wherein the small molecule modulator is an inhibitor of gene expression.

15. The method according to any of claims 1-14, wherein the gene acts on a transcription factor.

16. The method according to any of claims 1-14, wherein the gene acts on a transmembrane transporter.

17. The method according to claim 16, wherein the transmembrane transporter is a pump.

18. The method according to any of claims 1-14, wherein the gene acts on a kinase.

19. The method according to any of claims 1-14, wherein the gene acts on a G-protein.

20. The method according to any of claims 1-14, wherein the gene acts on a phosphatase.

21. The method according to any of claims 1-14, wherein the gene acts on a protease.

22. The method according to any of claims 1-14, wherein the gene acts on a biosynthetic enzyme.

23. The method according to any of claims 1-14, wherein the gene acts on a cell surface receptor.

24. The method according to any of claims 1-14, wherein the gene acts on a GTPase activating protein.

25. The method according to any of claims 1-14, wherein the gene acts on a guanine nucleotide exchange factor.

26. The method according to any of claims 1-14, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

27. The method according to any of claims 1-14, wherein the gene acts on a bacterial protein toxin.

28. The method according to any of claims 1-14, wherein the gene acts on an importin protein.

29. The method according to any of claims 1-14, wherein the gene acts on an RNA binding protein.

30. The method according to any of claims 1-14, wherein the gene acts on a component of a SCF complex.

31. The method according to any of claims 1-14, wherein the gene encodes a transcription factor.

32. The method according to any of claims 1-14, wherein the gene encodes a transmembrane transporter.

33. The method according to claim 32, wherein the transmembrane transporter is a pump.

34. The method according to any of claims 1-14, wherein the gene encodes a kinase.

35. The method according to any of claims 1-14, wherein the gene encodes a G-protein.

36. The method according to any of claims 1-14, wherein the gene encodes a phosphatase.

37. The method according to any of claims 1-14, wherein the gene encodes a protease.

38. The method according to any of claims 1-14, wherein the gene encodes a biosynthetic enzyme.

39. The method according to any of claims 1-14, wherein the gene encodes a cell surface receptor.

40. The method according to any of claims 1-14, wherein the gene encodes a GTPase activating protein.

41. The method according to any of claims 1-14, wherein the gene encodes a guanine nucleotide exchange factor.

42. The method according to any of claims 1-14, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

43. The method according to any of claims 1-14, wherein the gene encodes a bacterial protein toxin.

44. The method according to any of claims 1-14, wherein the gene encodes an importin protein.

45. The method according to any of claims 1-14, wherein the gene encodes an RNA binding protein.

46. The method according to any of claims 1-14, wherein the gene encodes a component of a SCF complex.

47. The method according to any of claims 1-19, wherein the secondary metabolite is an antibacterial.

48. The method according to claim 20, wherein the antibacterial is a ß-lactam.

49. The method according to claim 20, wherein the antibacterial is a cephalosporin.

50. The method according to any of claims 1-19, wherein the secondary metabolite is an anti-hypercholesterolemic.

51. The method according to claim 23, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

52. The method according to any of claims 1-46, wherein the secondary metabolite is an immunosuppressant.

53. The method according to claim 25, wherein the immunosuppressant is a cyclosporin.

54. The method according to any of claims 1-46, wherein the secondary metabolite is an ergot alkaloid.

55. The method according to any of claims 1-46, wherein the secondary metabolite is an angiogenesis inhibitor.

56. The method according to any of claims 1-46, wherein the secondary metabolite is a glucan synthesis inhibitor.

57. The method according to any of claims 1-46, wherein the secondary metabolite is a fungal toxin.

58. The method according to any of claims 1-46, wherein the secondary metabolite is a gliotoxin.

59. The method according to any of claims 1-46, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

60. The method according to any of claims 1-46, wherein the secondary metabolite is a plant growth regulator.

61. The method according to any of claims 1-46, wherein the secondary metabolite is a pigment.

62. The method according to any of claims 1-46, wherein the secondary metabolite is an insecticide.

63. The method according to any of claims 1-46, wherein the secondary metabolite is an anti-neoplastic compound.

64. The method according to any of claims 1-63, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

65. A method for improving production of a secondary metabolite by a fungus by increasing productivity of the secondary metabolite in the fungus, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that improves the productivity of the secondary metabolite, provided however, that when the secondary metabolite is isopenicillin N, then the modulation is not mediated by transcription factor CPCR1, and when the gene involved in regulation of secondary metabolite production is from *Saccharomyces cerevisiae*, then the modulation is not through decreased activity or expression of HOG1, BEM2, RIM15, SFL1, IRA1, SSD1, SRB11, SWI4, TPK3 or though increased activity or expression of AFL1, DHH1, INV7, INV8, STE21, PET9, MEP2, INV1, INV5, INV6, INV9, INV10, INV11, INV12, INV13, INV14, INV15, CDC25, MCM1, MGA1, PHD2, PHO23, PTC1, RIM1, STP22, TPK2, YPR1, or HAP4.

66. The method according to claim 65, wherein the modulation is overexpression of the gene.

67. The method according to claim 65, wherein the modulation is conditional expression of the gene.

68. The method according to claim 65, wherein the modulation is expression of a dominant mutation of the gene.

69. The method according to claim 68, wherein the dominant mutation is a dominant negative mutation.

70. The method according to claim 68, wherein the dominant mutation is a dominant neomorphic mutation.

71. The method according to claim 68, wherein the dominant mutation is a dominant positive mutation.

72. The method according to claim 65, wherein the modulation is mediated by a transcription factor.

73. The method according to claim 65, wherein the modulation is mediated by a peptide modulator of gene expression.

74. The method according to claim 73, wherein the peptide modulator is an activator of gene expression.

75. The method according to claim 73, wherein the peptide modulator is an inhibitor of gene expression.

76. The method according to claim 65, wherein the modulation is mediated by a small molecule modulator of gene expression.

77. The method according to claim 76, wherein the small molecule modulator is an activator of gene expression.

78. The method according to claim 76, wherein the small molecule modulator is an inhibitor of gene expression.

79. The method according to any of claims 65-78, wherein the gene acts on a transcription factor.

80. The method according to any of claims 65-78, wherein the gene acts on a transmembrane transporter.

81. The method according to any of claims 65-78, wherein the gene acts on a kinase.

82. The method according to any of claims 65-78, wherein the gene acts on a G-protein.

83. The method according to any of claims 65-78, wherein the gene acts on a phosphatase.

84. The method according to any of claims 65-78, wherein the gene acts on a protease.

85. The method according to any of claims 65-78, wherein the gene acts on a biosynthetic enzyme.

86. The method according to any of claims 65-78, wherein the gene acts on a cell surface receptor.

87. The method according to any of claims 65-86, wherein the gene acts on a GTPase activating protein.

88. The method according to any of claims 65-86, wherein the gene acts on a guanine nucleotide exchange factor.

89. The method according to any of claims 65-86, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

90. The method according to any of claims 65-86, wherein the gene acts on a bacterial protein toxin.

91. The method according to any of claims 65-86, wherein the gene acts on an importin protein.

92. The method according to any of claims 65-86, wherein the gene acts on an RNA binding protein.

93. The method according to any of claims 65-86, wherein the gene acts on a component of a SCF complex.

94. The method according to any of claims 65-86, wherein the gene encodes a transcription factor.

95. The method according to any of claims 65-86, wherein the gene encodes a transmembrane transporter.

96. The method according to claim 95, wherein the transmembrane transporter is a pump.

97. The method according to any of claims 65-86, wherein the gene encodes a kinase.

98. The method according to any of claims 65-86, wherein the gene encodes a G-protein.

99. The method according to any of claims 65-86, wherein the gene encodes a phosphatase.

100. The method according to any of claims 65-86, wherein the gene encodes a protease.

101. The method according to any of claims 65-86, wherein the gene encodes a biosynthetic enzyme.

102. The method according to any of claims 65-86, wherein the gene encodes a cell surface receptor.

103. The method according to any of claims 65-86, wherein the gene encodes a GTPase activating protein.

104. The method according to any of claims 65-86, wherein the gene encodes a guanine nucleotide exchange factor.

105. The method according to any of claims 65-86, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

106. The method according to any of claims 65-86, wherein the gene encodes a bacterial protein toxin.

107. The method according to any of claims 65-86, wherein the gene encodes an importin protein.

108. The method according to any of claims 65-86, wherein the gene encodes an RNA binding protein.

109. The method according to any of claims 65-86, wherein the gene encodes a component of a SCF complex.

110. The method according to any of claims 65-109, wherein the secondary metabolite is an antibacterial.

111. The method according to claim 110, wherein the antibacterial is a ß-lactam.

112. The method according to claim 110, wherein the antibacterial is a cephalosporin.

113. The method according to any of claims 65-109, wherein the secondary metabolite is an anti-hypercholesterolemic.

114. The method according to claim 113, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

115. The method according to any of claims 65-109, wherein the secondary metabolite is an immunosuppressant.

116. The method according to claim 115, wherein the immunosuppressant is a cyclosporin.

117. The method according to any of claims 65-109, wherein the secondary metabolite is an ergot alkaloid.

118. The method according to any of claims 65-109, wherein the secondary metabolite is an angiogenesis inhibitor.

119. The method according to any of claims 65-109, wherein the secondary metabolite is a glucan synthesis inhibitor.

120. The method according to any of claims 65-109, wherein the secondary metabolite is a fungal toxin.

121. The method according to any of claims 65-109, wherein the secondary metabolite is a gliotoxin.

122. The method according to any of claims 65-109, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

123. The method according to any of claims 65-109, wherein the secondary metabolite is a plant growth regulator.

124. The method according to any of claims 65-109, wherein the secondary metabolite is a pigment.

125. The method according to any of claims 65-109, wherein the secondary metabolite is an insecticide.

126. The method according to any of claims 65-109, wherein the secondary metabolite is an anti-neoplastic compound.

127. The method according to any of claims 65-126, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

128. A method for improving production of a secondary metabolite in a fungus by increasing efflux or excretion of the secondary metabolite, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that increases efflux or excretion the secondary metabolite.

129. The method according to claim 128, wherein the modulation is overexpression of the gene.

130. The method according to claim 128, wherein the modulation is conditional expression of the gene.

131. The method according to claim 128, wherein the modulation is expression of a dominant mutation of the gene.

132. The method according to claim 131, wherein the dominant mutation is a dominant negative mutation.

133. The method according to claim 131, wherein the dominant mutation is a dominant positive mutation.

134. The method according to claim 131, wherein the dominant mutation is a dominant neomorphic mutation.

135. The method according to claim 128, wherein the modulation is mediated by a transcription factor.

136. The method according to claim 128, wherein the modulation is mediated by a peptide modulator of gene expression.

137. The method according to claim 136, wherein the peptide modulator is an activator of gene expression.

138. The method according to claim 136, wherein the peptide modulator is an inhibitor of gene expression.

139. The method according to claim 128, wherein the modulation is mediated by a small molecule modulator of gene expression.

140. The method according to claim 139, wherein the small molecule modulator is an activator of gene expression.

141. The method according to claim 139, wherein the small molecule modulator is an inhibitor of gene expression.

142. The method according to any of claims 128-141, wherein the gene acts on a transcription factor.

143. The method according to any of claims 128-141, wherein the gene acts on a transmembrane transporter.

144. The method according to any of claims 128-141, wherein the gene acts on a kinase.

145. The method according to any of claims 128-141, wherein the gene acts on a G-protein.

146. The method according to any of claims 128-141, wherein the gene acts on a phosphatase.

147. The method according to any of claims 128-141, wherein the gene acts on a protease.

148. The method according to any of claims 128-141, wherein the gene acts on a biosynthetic enzyme.

149. The method according to any of claims 128-141, wherein the gene acts on a cell surface receptor.

150. The method according to any of claims 128-141, wherein the gene acts on a GTPase activating protein.

151. The method according to any of claims 128-141, wherein the gene acts on a guanine nucleotide exchange factor.

152. The method according to any of claims 128-141, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

153. The method according to any of claims 128-141, wherein the gene acts on a bacterial protein toxin.

154. The method according to any of claims 128-141, wherein the gene acts on an importin protein.

155. The method according to any of claims 128-141, wherein the gene acts on an RNA binding protein.

156. The method according to any of claims 128-141, wherein the gene acts on a component of a SCF complex.

157. The method according to any of claims 128-141, wherein the gene encodes a transcription factor.

158. The method according to any of claims 128-141, wherein the gene encodes a transmembrane transporter.

159. The method according to any of claim 158, wherein the transmembrane transporter is a pump.

160. The method according to any of claims 128-141, wherein the gene encodes a kinase.

161. The method according to any of claims 128-141, wherein the gene encodes a G-protein.

162. The method according to any of claims 128-141, wherein the gene encodes a phosphatase.

163. The method according to any of claims 128-141, wherein the gene encodes a protease.

164. The method according to any of claims 128-141, wherein the gene encodes a biosynthetic enzyme.

165. The method according to any of claims 128-141, wherein the gene encodes a cell surface receptor.

166. The method according to any of claims 128-141, wherein the gene encodes a GTPase activating protein.

167. The method according to any of claims 128-141, wherein the gene encodes a guanine nucleotide exchange factor.

168. The method according to any of claims 128-141, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

169. The method according to any of claims 128-141, wherein the gene encodes a bacterial protein toxin.

170. The method according to any of claims 128-141, wherein the gene encodes an importin protein.

171. The method according to any of claims 128-141, wherein the gene encodes an RNA binding protein.

172. The method according to any of claims 128-141, wherein the gene encodes a component of a SCF complex.

173. The method according to any of claims 128-172, wherein the secondary metabolite is an antibacterial.

174. The method according to claim 173, wherein the antibacterial is a ß-lactam.

175. The method according to claim 173, wherein the antibacterial is a cephalosporin.

176. The method according to any of claims 128-172, wherein the secondary metabolite is an anti-hypercholesterolemic.

177. The method according to claim 176, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

178. The method according to any of claims 128-172, wherein the secondary metabolite is an immunosuppressant.

179. The method according to claim 178, wherein the immunosuppressant is a cyclosporin.

180. The method according to any of claims 128-172, wherein the secondary metabolite is an ergot alkaloid.

181. The method according to any of claims 128-172, wherein the secondary metabolite is an angiogenesis inhibitor.

182. The method according to any of claims 128-172, wherein the secondary metabolite is a glucan synthesis inhibitor.

183. The method according to any of claims 128-172, wherein the secondary metabolite is a fungal toxin.

184. The method according to any of claims 128-172, wherein the secondary metabolite is a gliotoxin.

185. The method according to any of claims 128-172, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

186. The method according to any of claims 128-172, wherein the secondary metabolite is a plant growth regulator.

187. The method according to any of claims 128-172, wherein the secondary metabolite is a pigment.

188. The method according to any of claims 128-172, wherein the secondary metabolite is an insecticide.

189. The method according to any of claims 128-172, wherein the secondary metabolite is an anti-neoplastic compound.

190. The method according to any of claims 128-189, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

191. A method for improving production of a secondary metabolite in a fungus by decreasing production of side products or competing secondary metabolites, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that decreases production of side products or competing secondary metabolites.

192. The method according to claim 191, wherein the modulation is overexpression of the gene.

193. The method according to claim 191, wherein the modulation is conditional expression of the gene.

194. The method according to claim 191, wherein the modulation is expression of a dominant mutation of the gene.

195. The method according to claim 194, wherein the dominant mutation is a dominant negative mutation.

196. The method according to claim 194, wherein the dominant mutation is a dominant positive mutation.

197. The method according to claim 194, wherein the dominant mutation is a dominant neomorphic mutation.

198. The method according to claim 191, wherein the modulation is mediated by a transcription factor.

199. The method according to claim 191, wherein the modulation is mediated by a peptide modulator of gene expression.

200. The method according to claim 199, wherein the peptide modulator is an activator of gene expression.

201. The method according to claim 199, wherein the peptide modulator is an inhibitor of gene expression.

202. The method according to claim 191, wherein the modulation is mediated by a small molecule modulator of gene expression.

203. The method according to claim 202, wherein the small molecule modulator is an activator of gene expression.

204. The method according to claim 202, wherein the small molecule modulator is an inhibitor of gene expression.

205. The method according to any of claims 191-204, wherein the gene acts on a transcription factor.

206. The method according to any of claims 191-204, wherein the gene encodes a transmembrane transporter.

207. The method according to any of claims 191-204, wherein the gene acts on a kinase.

208. The method according to any of claims 85-96, wherein the gene acts on a G-protein.

209. The method according to any of claims 191-204, wherein the gene acts on a phosphatase.

210. The method according to any of claims 191-204, wherein the gene acts on a protease.

211. The method according to any of claims 191-204, wherein the gene acts on a biosynthetic enzyme.

212. The method according to any of claims 191-204, wherein the gene acts on a cell surface receptor.

213. The method according to any of claims 191-204, wherein the gene acts on a GTPase activating protein.

214. The method according to any of claims 191-204, wherein the gene acts on a guanine nucleotide exchange factor.

215. The method according to any of claims 191-204, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

216. The method according to any of claims 191-204, wherein the gene acts on a bacterial protein toxin.

217. The method according to any of claims 191-204, wherein the gene acts on an importin protein.

218. The method according to any of claims 191-204, wherein the gene acts on an RNA binding protein.

219. The method according to any of claims 191-204, wherein the gene acts on a component of a SCF complex.

220. The method according to any of claims 191-204, wherein the gene encodes a transcription factor.

221. The method according to any of claims 191-204, wherein the gene encodes a transmembrane transporter.

222. The method according to claim 221, wherein the transmembrane transporter is a pump.

223. The method according to any of claims 191-204, wherein the gene encodes a kinase.

224. The method according to any of claims 191-204, wherein the gene encodes a G-protein.

225. The method according to any of claims 191-204, wherein the gene encodes a phosphatase.

226. The method according to any of claims 191-204, wherein the gene encodes a protease.

227. The method according to any of claims 191-204, wherein the gene encodes a biosynthetic enzyme.

228. The method according to any of claims 191-204, wherein the gene encodes a cell surface receptor.

229. The method according to any of claims 191-204, wherein the gene encodes a GTPase activating protein.

230. The method according to any of claims 191-204, wherein the gene encodes a guanine nucleotide exchange factor.

231. The method according to any of claims 191-204, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

232. The method according to any of claims 191-204, wherein the gene encodes a bacterial protein toxin.

233. The method according to any of claims 191-204, wherein the gene encodes an importin protein.

234. The method according to any of claims 191-204, wherein the gene encodes an RNA binding protein.

235. The method according to any of claims 191-204, wherein the gene encodes a component of a SCF complex.

236. The method according to any of claims 191-235, wherein the secondary metabolite is an antibacterial.

237. The method according to claim 236, wherein the antibacterial is a ß-lactam.

238. The method according to claim 236, wherein the antibacterial is a cephalosporin.

239. The method according to any of claims 191-235, wherein the secondary metabolite is an anti-hypercholesterolemic.

240. The method according to claim 239, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

241. The method according to any of claims 191-235, wherein the secondary metabolite is an immunosuppressant.

242. The method according to claim 241, wherein the immunosuppressant is a cyclosporin.

243. The method according to any of claims 191-235, wherein the secondary metabolite is an ergot alkaloid.

244. The method according to any of claims 191-235, wherein the secondary metabolite is an angiogenesis inhibitor.

245. The method according to any of claims 191-235, wherein the secondary metabolite is a glucan synthesis inhibitor.

246. The method according to any of claims 191-235, wherein the secondary metabolite is a fungal toxin.

247. The method according to any of claims 191-235, wherein the secondary metabolite is a gliotoxin.

248. The method according to any of claims 191-235, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

249. The method according to any of claims 191-235, wherein the secondary metabolite is a plant growth regulator.

250. The method according to any of claims 191-235, wherein the secondary metabolite is a pigment.

251. The method according to any of claims 191-235, wherein the secondary metabolite is an insecticide.

252. The method according to any of claims 191-235, wherein the secondary metabolite is an anti-neoplastic compound.

253. The method according to any of claims 191-252, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

254. A method for improving production of a secondary metabolite in a fungus by altering the characteristics of the fungus in a manner that is beneficial to the production of the secondary metabolite, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that alters the characteristics of the fungus.

255. The method according to claim 254, wherein the altered characteristic is transition from hyphal growth to yeast form.

256. The method according to claim 254, wherein the altered characteristic is an increase or decrease in flocculence.

257. The method according to claim 254, wherein the altered characteristic is increased or decreased adhesion to a surface.

258. The method according to any of claims 254-257, wherein the modulation is overexpression of the gene.

259. The method according to any of claims 254-257, wherein the modulation is conditional expression of the gene.

260. The method according to any of claims 254-257, wherein the modulation is expression of a dominant mutation of the gene.

261. The method according to claim 260, wherein the dominant mutation is a dominant negative mutation.

262. The method according to claim 260, wherein the dominant mutation is a dominant positive mutation.

263. The method according to claim 260, wherein the dominant mutation is a dominant neomorphic mutation.

264. The method according to any of claims 254-257, wherein the modulation is mediated by a transcription factor.

265. The method according to any of claims 254-257, wherein the modulation is mediated by a peptide modulator of gene expression.

266. The method according to claim 265, wherein the peptide modulator is an activator of gene expression.

267. The method according to claim 265, wherein the peptide modulator is an inhibitor of gene expression.

268. The method according to any of claims 254-257, wherein the modulation is mediated by a small molecule modulator of gene expression.

269. The method according to claim 268, wherein the small molecule modulator is an activator of gene expression.

270. The method according to claim 268, wherein the small molecule modulator is an inhibitor of gene expression.

271. The method according to any of claims 254-270, wherein the gene acts on a transcription factor.

272. The method according to any of claims 254-270, wherein the gene acts on a transmembrane transporter.

273. The method according to any of claims 254-270, wherein the gene acts on a kinase.

274. The method according to any of claims 254-270, wherein the gene acts on a G-protein.

275. The method according to any of claims 254-270, wherein the gene acts on a phosphatase.

276. The method according to any of claims 254-270, wherein the gene acts on a protease.

277. The method according to any of claims 254-270, wherein the gene acts on a biosynthetic enzyme.

278. The method according to any of claims 254-270, wherein the gene acts on a cell surface receptor.

279. The method according to any of claims 254-270, wherein the gene acts on a GTPase activating protein.

280. The method according to any of claims 254-270, wherein the gene acts on a guanine nucleotide exchange factor.

281. The method according to any of claims 254-270, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

282. The method according to any of claims 254-270, wherein the gene acts on a bacterial protein toxin.

283. The method according to any of claims 254-270, wherein the gene acts on an importin protein.

284. The method according to any of claims 254-270, wherein the gene acts on an RNA binding protein.

285. The method according to any of claims 254-270, wherein the gene acts on a component of a SCF complex.

286. The method according to any of claims 254-270, wherein the gene encodes a transcription factor.

287. The method according to any of claims 254-270, wherein the gene encodes a transmembrane transporter.

288. The method according to claim 287, wherein the transmembrane transporter is a pump.

289. The method according to any of claims 254-270, wherein the gene encodes a kinase.

290. The method according to any of claims 254-270, wherein the gene encodes a G-protein.

291. The method according to any of claims 254-270, wherein the gene encodes a phosphatase.

292. The method according to any of claims 254-270, wherein the gene encodes a protease.

293. The method according to any of claims 254-270, wherein the gene encodes a biosynthetic enzyme.

294. The method according to any of claims 254-270, wherein the gene encodes a cell surface receptor.

295. The method according to any of claims 254-270, wherein the gene encodes a GTPase activating protein.

296. The method according to any of claims 254-270, wherein the gene encodes a guanine nucleotide exchange factor.

297. The method according to any of claims 254-270, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

298. The method according to any of claims 254-270, wherein the gene encodes a bacterial protein toxin.

299. The method according to any of claims 254-270, wherein the gene encodes an importin protein.

300. The method according to any of claims 254-270, wherein the gene encodes an RNA binding protein.

301. The method according to any of claims 254-270, wherein the gene encodes a component of a SCF complex.

302. The method according to any of claims 254-301, wherein the secondary metabolite is an antibacterial.

303. The method according to claim 302, wherein the antibacterial is a ß-lactam.

304. The method according to claim 302, wherein the antibacterial is a cephalosporin.

305. The method according to any of claims 254-301, wherein the secondary metabolite is an anti-hypercholesterolemic.

306. The method according to claim 305, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

307. The method according to any of claims 254-301, wherein the secondary metabolite is an immunosuppressant.

308. The method according to claim 307, wherein the immunosuppressant is a cyclosporin.

309. The method according to any of claims 254-301, wherein the secondary metabolite is an ergot alkaloid.

310. The method according to any of claims 254-301, wherein the secondary metabolite is an angiogenesis inhibitor.

311. The method according to any of claims 254-301, wherein the secondary metabolite is a glucan synthesis inhibitor.

312. The method according to any of claims 254-301, wherein the secondary metabolite is a fungal toxin.

313. The method according to any of claims 254-301, wherein the secondary metabolite is a gliotoxin.

314. The method according to any of claims 254-301, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

315. The method according to any of claims 254-301, wherein the secondary metabolite is a plant growth regulator.

316. The method according to any of claims 254-301, wherein the secondary metabolite is a pigment.

317. The method according to any of claims 254-301, wherein the secondary metabolite is an insecticide.

318. The method according to any of claims 254-301, wherein the secondary metabolite is an anti-neoplastic compound.

319. The method according to any of claims 254-318, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

320. A method for improving production of a secondary metabolite in a fungus by causing conditional lysis of the fungus, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that causes conditional lysis.

321. The method according to claim 320, wherein the modulation is overexpression of the gene.

322. The method according to claim 320, wherein the modulation is conditional expression of the gene.

323. The method according to claim 320, wherein the modulation is expression of a dominant mutation of the gene.

324. The method according to claim 323, wherein the dominant mutation is a dominant negative mutation.

325. The method according to claim 323, wherein the dominant mutation is a dominant positive mutation.

326. The method according to claim 323, wherein the dominant mutation is a dominant neomorphic mutation.

327. The method according to claim 320, wherein the modulation is mediated by a transcription factor.

328. The method according to claim 320, wherein the modulation is mediated by a peptide modulator of gene expression.

329. The method according to claim 328, wherein the peptide modulator is an activator of gene expression.

330. The method according to claim 328, wherein the peptide modulator is an inhibitor of gene expression.

331. The method according to claim 320, wherein the modulation is mediated by a small molecule modulator of gene expression.

332. The method according to claim 331, wherein the small molecule modulator is an activator of gene expression.

333. The method according to claim 331, wherein the small molecule modulator is an inhibitor of gene expression.

334. The method according to any of claims 320-333, wherein the gene acts on a transcription factor.

335. The method according to any of claims 320-333, wherein the gene acts on a transmembrane transporter.

336. The method according to any of claims 320-333, wherein the gene acts on a kinase.

337. The method according to any of claims 320-333, wherein the gene acts on a G-protein.

338. The method according to any of claims 320-333, wherein the gene acts on a phosphatase.

339. The method according to any of claims 320-333, wherein the gene acts on a protease.

340. The method according to any of claims 320-333, wherein the gene acts on a biosynthetic enzyme.

341. The method according to any of claims 320-333, wherein the gene acts on a cell surface receptor.

342. The method according to any of claims 320-333, wherein the gene acts on a GTPase activating protein.

343. The method according to any of claims 320-333, wherein the gene acts on a guanine nucleotide exchange factor.

344. The method according to any of claims 320-333, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

345. The method according to any of claims 320-333, wherein the gene acts on a bacterial protein toxin.

346. The method according to any of claims 320-333, wherein the gene acts on an importin protein.

347. The method according to any of claims 320-333, wherein the gene acts on an RNA binding protein.

348. The method according to any of claims 320-333, wherein the gene acts on a component of a SCF complex.

349. The method according to any of claims 320-333, wherein the gene encodes a transcription factor.

350. The method according to any of claims 320-333, wherein the gene encodes a transmembrane transporter.

351. The method according to claim 350, wherein the transmembrane transporter is a pump.

352. The method according to any of claims 320-333, wherein the gene encodes a kinase.

353. The method according to any of claims 320-333, wherein the gene encodes a G-protein.

354. The method according to any of claims 320-333, wherein the gene encodes a phosphatase.

355. The method according to any of claims 320-333, wherein the gene encodes a protease.

356. The method according to any of claims 320-333, wherein the gene encodes a biosynthetic enzyme.

357. The method according to any of claims 320-333, wherein the gene encodes a cell surface receptor.

358. The method according to any of claims 320-333, wherein the gene encodes a GTPase activating protein.

359. The method according to any of claims 320-333, wherein the gene encodes a guanine nucleotide exchange factor.

360. The method according to any of claims 320-333, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

361. The method according to any of claims 320-333, wherein the gene encodes a bacterial protein toxin.

362. The method according to any of claims 320-333, wherein the gene encodes an importin protein.

363. The method according to any of claims 320-333, wherein the gene encodes an RNA binding protein.

364. The method according to any of claims 320-333, wherein the gene encodes a component of a SCF complex.

365. The method according to any of claims 320-364, wherein the secondary metabolite is an antibacterial.

366. The method according to claim 365, wherein the antibacterial is a ß-lactam.

367. The method according to claim 365, wherein the antibacterial is a cephalosporin.

368. The method according to any of claims 320-364, wherein the secondary metabolite is an anti-hypercholesterolemic.

369. The method according to claim 368, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

370. The method according to any of claims 320-364, wherein the secondary metabolite is an immunosuppressant.

371. The method according to claim 370, wherein the immunosuppressant is a cyclosporin.

372. The method according to any of claims 320-364, wherein the secondary metabolite is an ergot alkaloid.

373. The method according to any of claims 320-364, wherein the secondary metabolite is an angiogenesis inhibitor.

374. The method according to any of claims 320-364, wherein the secondary metabolite is a glucan synthesis inhibitor.

375. The method according to any of claims 320-364, wherein the secondary metabolite is a fungal toxin.

376. The method according to any of claims 320-364, wherein the secondary metabolite is a gliotoxin.

377. The method according to any of claims 320-364, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

378. The method according to any of claims 320-364, wherein the secondary metabolite is a plant growth regulator.

379. The method according to any of claims 320-364, wherein the secondary metabolite is a pigment.

380. The method according to any of claims 320-364, wherein the secondary metabolite is an insecticide.

381. The method according to any of claims 320-364, wherein the secondary metabolite is an anti-neoplastic compound.

382. The method according to any of claims 320-381, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

383. A method for improving production of a secondary metabolite in a fungus by increasing the resistance of the fungus to the deleterious effects of exposure to a secondary metabolite, the method comprising modulating the expression of a gene involved in regulation of secondary metabolite production in a manner that increases resistance to the deleterious effects of exposure to a secondary metabolite.

384. The method according to claim 383, wherein the modulation is overexpression of the gene.

385. The method according to claim 383, wherein the modulation is conditional expression of the gene.

386. The method according to claim 383, wherein the modulation is expression of a dominant mutation of the gene.

387. The method according to claim 386, wherein the dominant mutation is a dominant negative mutation.

388. The method according to claim 386, wherein the dominant mutation is a dominant positive mutation.

389. The method according to claim 386, wherein the dominant mutation is a dominant neomorphic mutation.

390. The method according to claim 383, wherein the modulation is mediated by a transcription factor.

391. The method according to claim 383, wherein the modulation is mediated by a peptide modulator of gene expression.

392. The method according to claim 391, wherein the peptide modulator is an activator of gene expression.

393. The method according to claim 391, wherein the peptide modulator is an inhibitor of gene expression.

394. The method according to claim 383, wherein the modulation is mediated by a small molecule modulator of gene expression.

395. The method according to claim 395, wherein the small molecule modulator is an activator of gene expression.

396. The method according to claim 395, wherein the small molecule modulator is an inhibitor of gene expression.

397. The method according to any of claims 383-396, wherein the gene acts on a transcription factor.

398. The method according to any of claims 383-396, wherein the gene acts on a transmembrane transporter.

399. The method according to any of claims 383-396, wherein the gene acts on a kinase.

400. The method according to any of claims 383-396, wherein the gene acts on a G-protein.

401. The method according to any of claims 383-396, wherein the gene acts on a phosphatase.

402. The method according to any of claims 383-396, wherein the gene acts on a protease.

403. The method according to any of claims 383-396, wherein the gene acts on a biosynthetic enzyme.

404. The method according to any of claims 383-396, wherein the gene acts on a cell surface receptor.

405. The method according to any of claims 383-396, wherein the gene acts on a GTPase activating protein.

406. The method according to any of claims 383-396, wherein the gene acts on a guanine nucleotide exchange factor.

407. The method according to any of claims 383-396, wherein the gene acts on a cyclic nucleotide phosphodiesterase.

408. The method according to any of claims 383-396, wherein the gene acts on a bacterial protein toxin.

409. The method according to any of claims 383-396, wherein the gene acts on an importin protein.

410. The method according to any of claims 383-396, wherein the gene acts on an RNA binding protein.

411. The method according to any of claims 383-396, wherein the gene acts on a component of a SCF complex.

412. The method according to any of claims 383-396, wherein the gene encodes a transcription factor.

413. The method according to any of claims 383-396, wherein the gene encodes a transmembrane transporter.

414. The method according to claim 413 wherein the transmembrane transporter is a pump.

415. The method according to any of claims 383-396, wherein the gene encodes a kinase.

416. The method according to any of claims 383-396, wherein the gene encodes a G-protein.

417. The method according to any of claims 383-396, wherein the gene encodes a phosphatase.

418. The method according to any of claims 383-396, wherein the gene encodes a protease.

419. The method according to any of claims 383-396, wherein the gene encodes a biosynthetic enzyme.

420. The method according to any of claims 383-396, wherein the gene encodes a cell surface receptor.

421. The method according to any of claims 383-396, wherein the gene encodes a GTPase activating protein.

422. The method according to any of claims 383-396, wherein the gene encodes a guanine nucleotide exchange factor.

423. The method according to any of claims 383-396, wherein the gene encodes a cyclic nucleotide phosphodiesterase.

424. The method according to any of claims 383-396, wherein the gene encodes a bacterial protein toxin.

425. The method according to any of claims 383-396, wherein the gene encodes an importin protein.

426. The method according to any of claims 383-396, wherein the gene encodes an RNA binding protein.

427. The method according to any of claims 383-396, wherein the gene encodes a component of a SCF complex.

428. The method according to any of claims 383-427, wherein the secondary metabolite is an antibacterial.

429. The method according to claim 428, wherein the antibacterial is a ß-lactam.

430. The method according to claim 428, wherein the antibacterial is a cephalosporin.

431. The method according to any of claims 383-427, wherein the secondary metabolite is an anti-hypercholesterolemic.

432. The method according to claim 431, wherein the anti-hypercholesterolemic is selected from lovastatin, mevastatin, simvastatin and pravastatin.

433. The method according to any of claims 383-427, wherein the secondary metabolite is an immunosuppressant.

434. The method according to claim 433, wherein the immunosuppressant is a cyclosporin.

435. The method according to any of claims 383-427, wherein the secondary metabolite is an ergot alkaloid.

436. The method according to any of claims 383-427, wherein the secondary metabolite is an angiogenesis inhibitor.

437. The method according to any of claims 383-427, wherein the secondary metabolite is a glucan synthesis inhibitor.

438. The method according to any of claims 383-427, wherein the secondary metabolite is a fungal toxin.

439. The method according to any of claims 383-427, wherein the secondary metabolite is a gliotoxin.

440. The method according to any of claims 383-427, wherein the secondary metabolite is a modulator of cell surface receptor signaling.

441. The method according to any of claims 383-427, wherein the secondary metabolite is a plant growth regulator.

442. The method according to any of claims 383-427, wherein the secondary metabolite is a pigment.

443. The method according to any of claims 383-427, wherein the secondary metabolite is an insecticide.

444. The method according to any of claims 383-427, wherein the secondary metabolite is an anti-neoplastic compound.

445. The method according to any of claims 383-444, further comprising the step of purifying the secondary metabolite from a culture of the fungus.

446. A genetically modified fungus, wherein the genetically modified fungus has an ability to produce secondary metabolites and the ability of the genetically modified fungus to produce secondary metabolites has been improved by any of the methods of claims 1-445.

447. A method for making a secondary metabolite, the method comprising culturing a genetically modified fungus according to claim 446 under conditions suitable for the production of secondary metabolites.

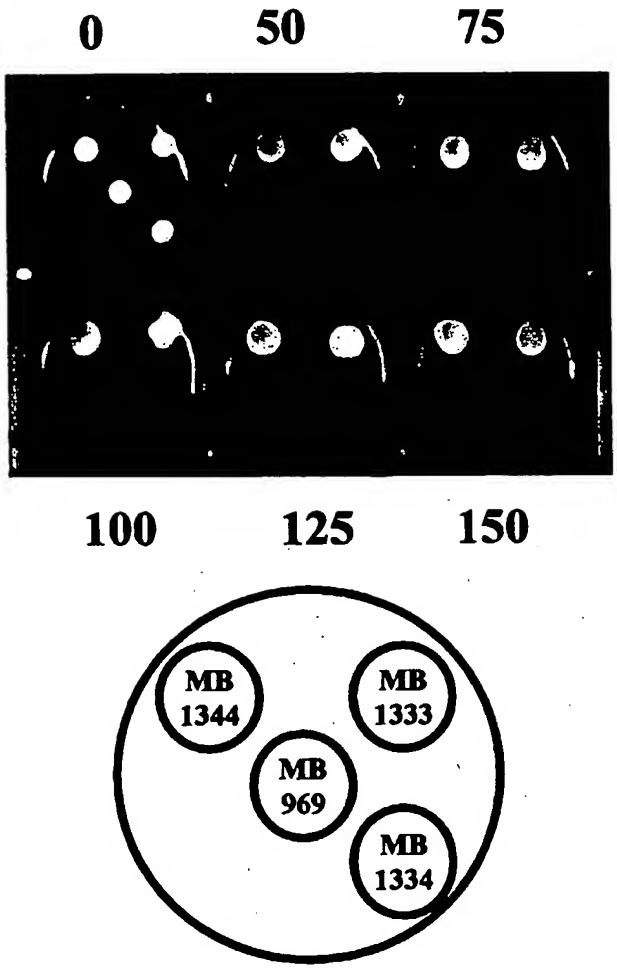


FIG. 1

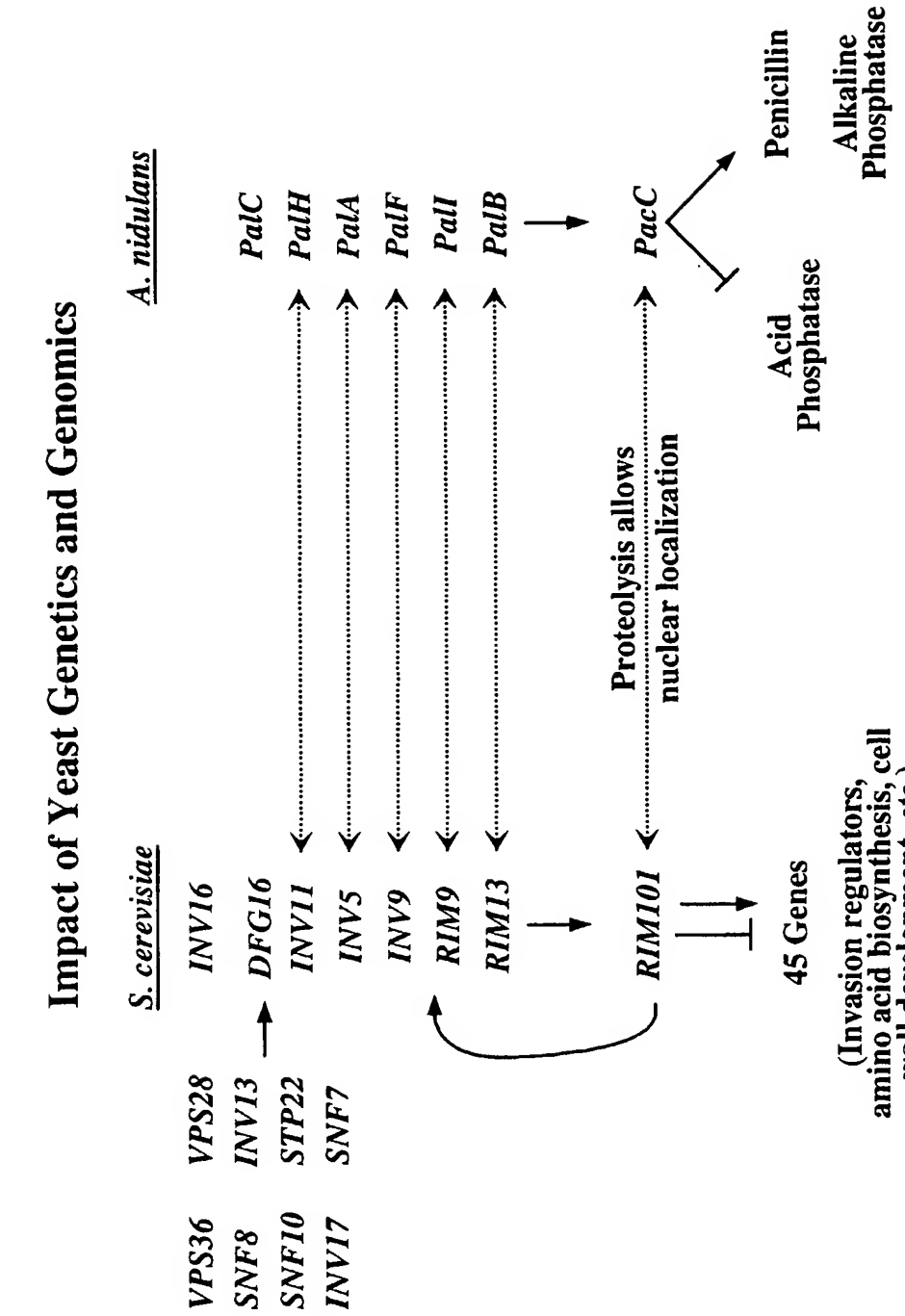

Impact of Yeast Genetics and Genomics
S. cerevisiae
A. nidulans
VPS36
SNF8
SNF10
INV17
VPS28
INV13
STP22
SNF7
INV16
DFG16
INV11
INV5
INV9
RIM9
RIM13
RIM101
PalC
PalH
PalA
PalF
PalI
PalB
PacC
Proteolysis allows
nuclear localization
45 Genes
(Invasion regulators,
amino acid biosynthesis, cell
wall development, etc.)
Acid
Phosphatase
Penicillin
Alkaline
Phosphatase

FIG. 2

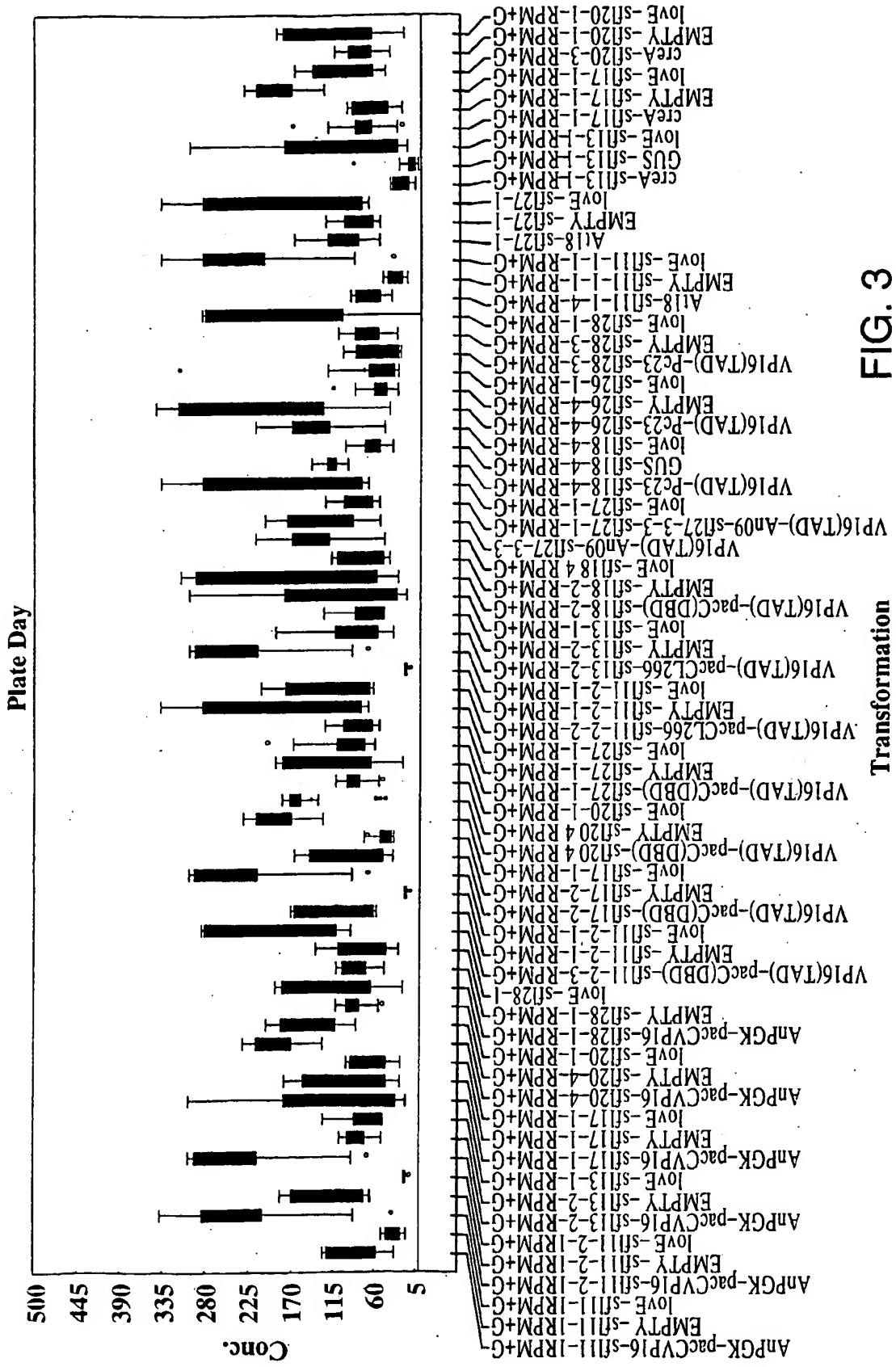


Plate Day
Conc.
500
445
390
335
280
225
170
115
60
5
Transformation
AnPGK-pacCVP16-sfI11-1RPM+G
EMPTY -sfI11-1RPM+G
lovE -sfI11-1RPM+G
AnPGK-pacCVP16-sfI11-2-1RPM+G
EMPTY -sfI11-2-1RPM+G
lovE -sfI11-2-1RPM+G
AnPGK-pacCVP16-sfI13-2-RPM+G
EMPTY -sfI13-2-RPM+G
lovE -sfI13-1-RPM+G
AnPGK-pacCVP16-sfI17-1-RPM+G
EMPTY -sfI17-1-RPM+G
lovE -sfI17-1-RPM+G
AnPGK-pacCVP16-sfI20-4-RPM+G
EMPTY -sfI20-4-RPM+G
lovE -sfI20-1-RPM+G
AnPGK-pacCVP16-sfI28-1-RPM+G
EMPTY -sfI28-1-RPM+G
lovE -sfI28-1
VP16(TAD)-pacC(DBD)-sfI11-2-3-RPM+G
EMPTY -sfI11-2-1-RPM+G
lovE -sfI11-2-1-RPM+G
VP16(TAD)-pacC(DBD)-sfI17-2-RPM+G
EMPTY -sfI17-2-RPM+G
lovE -sfI17-1-RPM+G
VP16(TAD)-pacC(DBD)-sfI20 4 RPM+G
EMPTY -sfI20 4 RPM+G
lovE -sfI20-1-RPM+G
VP16(TAD)-pacC(DBD)-sfI27-1-RPM+G
EMPTY -sfI27-1-RPM+G
lovE -sfI27-1-RPM+G
VP16(TAD)-pacCL266-sfI11-2-2-RPM+G
EMPTY -sfI11-2-1-RPM+G
lovE -sfI11-2-1-RPM+G
VP16(TAD)-pacCL266-sfI13-2-RPM+G
EMPTY -sfI13-2-RPM+G
lovE -sfI13-1-RPM+G
VP16(TAD)-pacC(DBD)-sfI18-2-RPM+G
EMPTY -sfI18-2-RPM+G
lovE -sfI18 4 RPM+G
VP16(TAD)-An09-sfI27-3-3
VP16(TAD)-An09-sfI27-3-3-sfI27-1-RPM+G
lovE -sfI27-1-RPM+G
VP16(TAD)-pc23-sfI18-4-RPM+G
GUS-sfI18-4-RPM+G
lovE -sfI18-4-RPM+G
VP16(TAD)-pc23-sfI26-4-RPM+G
EMPTY -sfI26-4-RPM+G
lovE -sfI26-1-RPM+G
VP16(TAD)-pc23-sfI28-3-RPM+G
EMPTY -sfI28-3-RPM+G
lovE -sfI28-1-RPM+G
At18-sfI11-1-4-RPM+G
EMPTY -sfI11-1-1-RPM+G
lovE -sfI11-1-1-RPM+G
At18-sfI27-1
EMPTY -sfI27-1
lovE -sfI27-1
creA-sfI13-1RPM+G
GUS -sfI13-1RPM+G
lovE -sfI13-1RPM+G
creA-sfI17-1-RPM+G
EMPTY -sfI17-1-RPM+G
lovE -sfI17-1-RPM+G
creA-sfI20-3-RPM+G
EMPTY -sfI20-1-RPM+G
lovE -sfI20-1-RPM+G

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/28903

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/37 C12N15/80 C12P21/00 C12R1/645

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N C12P C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 25735 A (MICROBIA INC) 27 May 1999 (1999-05-27) the whole document	1,446, 447
X	WO 99 25865 A (MICROBIA INC) 27 May 1999 (1999-05-27) the whole document	1,446, 447
X	EP 0 357 119 A (GIST BROCADES NV) 7 March 1990 (1990-03-07) the whole document, in particular page 8 line 52 to page 9 lines 3	1,446, 447
X	US 5 665 543 A (LEICHTFRIED FRANZ ET AL) 9 September 1997 (1997-09-09) the whole document, in particular columns 31-34	1,446, 447

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 March 2001	21.03.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Julia, P

4

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/28903

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 00 37629 A (WISCONSIN ALUMNI RES FOUND) 29 June 2000 (2000-06-29) cited in the application the whole document -----	1,446, 447

4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/28903

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2-445 (complete); 1, 446-447 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 2-445 (complete); 1, 446-447 (partially)

In view of the large number of claims present on file as well as their wording, the International Search Agency considers that it is difficult, if not impossible, to determine the matter for which protection is actually sought and that the present application fails to comply with the clarity and conciseness requirements of Article 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible.

Moreover, the claims relate to methods defined only by reference to a desirable characteristic, namely "... modulating the expression of a gene involved in regulation of secondary metabolite production in a manner..." that (i) improves the yield of the secondary metabolite (claims 1-64), (ii) improves the productivity of the secondary metabolite (claims 65-127), (iii) increases the flux or excretion of the secondary metabolite, (claims 128-190), (iv) decreases the production of side products or competing secondary metabolites (claims 191-253), (v) alters the characteristics of the fungus (claims 254-319), (vi) causes conditional lysis (claims 320-382) and (vii) increases resistance to the deleterious effects of exposure to a secondary metabolite (claims 383-445) as well as to related products (genetically modified fungi, claim 446) and uses thereof (method of making a secondary metabolite, claim 447). The claims cover all methods having this characteristic, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such methods. In the present case, the claims so lack support and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the methods by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Furthermore, according to Rule 13 PCT an application must relate to one invention only or to a group of inventions so linked as to form a single general inventive concept, i.e. having at least one common technical feature defining a contribution over the known prior art. In the present case, the common technical feature among the different groups of inventions seems to be "...the modulation of the expression of a gene involved in the regulation of secondary metabolite production ... for improving the production of a secondary metabolite by a fungus ...". However, this concept was already known in the prior art (see disclaimers in claim 1, bibliographic references in the description of the application as well as the partial ISR). Therefore, in principle seven different groups of inventions could be identified corresponding to the seven independent claims of the present application, namely methods for improving the production of a secondary metabolite modulating the expression of a gene involved in the regulation of secondary metabolite production in a manner that : (i) improves the yield of the secondary metabolite (claims 1-64), (ii) improves the productivity of the secondary metabolite (claims 65-127), (iii) increases efflux or excretion of the

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

secondary metabolite (claims 128-190), (iv) decreases the production of side products or competing secondary metabolites (claims 191-253), (v) alters the characteristics of the fungus (claims 254-319), (vi) causes conditional lysis (claims 320-382), (vii) increases resistance to the deleterious effects of exposure to a secondary metabolite (claims 383-445) as well as related genetically modified fungi and uses thereof for each group (claims 446-447, partially). However, in view of the references cited in the application and in the partial ISR, the ISA fails to see which is the single inventive concept among the different embodimens corresponding to the depent claims of each group of inventions. Thus, each and every group identified above comprises several subgroups of inventions, such as : (i) type of modulation and/or modulator, (ii) type and effects of the genes involved in the regulation of the secondary metabolite production, (iii) type of secondary metabolite, etc...

In conclusion, the claims do not fulfil the requirements of Articles 5 and 6 PCT and Rule 13 PCT. A meaningful search over the whole of the claimed scope is impossible and consequently, the search has been carried out only for those parts of the application which do appear to be (i) clear, concise, technically supported and (ii) which correspond to the first group of invention (claims 1 and 446-447, partially), namely those parts relating to methods for increasing the yield of a secondary metabolite using the "fungal regulators" of Table 1 (examples 3-5). However, in view of the document WO99/25735 no common inventive concept seems to be present among these several fungal regulators.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/28903

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9925735 A	27-05-1999	AU 1529099 A	07-06-1999
		AU 1797999 A	07-06-1999
		EP 1049797 A	08-11-2000
		EP 1032590 A	06-09-2000
		WO 9925865 A	27-05-1999
WO 9925865 A	27-05-1999	AU 1529099 A	07-06-1999
		AU 1797999 A	07-06-1999
		EP 1049797 A	08-11-2000
		EP 1032590 A	06-09-2000
		WO 9925735 A	27-05-1999
EP 0357119 A	07-03-1990	AT 130033 T	15-11-1995
		AU 631371 B	26-11-1992
		AU 3956889 A	15-02-1990
		AU 631806 B	10-12-1992
		AU 3956989 A	15-02-1990
		DE 68924745 D	14-12-1995
		DK 393089 A	12-02-1990
		DK 393189 A	12-02-1990
		EP 0354624 A	14-02-1990
		ES 2081831 T	16-03-1996
		FI 893722 A	12-02-1990
		FI 893723 A	12-02-1990
		GR 3018782 T	30-04-1996
		HU 51335 A,B	28-04-1990
		IE 72167 B	26-03-1997
		JP 2167078 A	27-06-1990
		JP 2954601 B	27-09-1999
		JP 2150284 A	08-06-1990
		JP 3095391 B	03-10-2000
		JP 2000342289 A	12-12-2000
		PT 91410 A,B	08-03-1990
		PT 91411 A,B	08-03-1990
		US 5462862 A	31-10-1995
		US 5747285 A	05-05-1998
		US 5108918 A	28-04-1992
US 5665543 A	09-09-1997	US 5976793 A	02-11-1999
		US 6136779 A	24-10-2000
		US 5863733 A	26-01-1999
		US 6165712 A	26-12-2000
		US 5846720 A	08-12-1998
		US 5580722 A	03-12-1996
		AU 660405 B	29-06-1995
		AU 6140090 A	22-02-1991
		CA 2063822 A	19-01-1991
		EP 0483249 A	06-05-1992
		JP 4506902 T	03-12-1992
		WO 9101379 A	07-02-1991
WO 0037629 A	29-06-2000	AU 2180100 A	12-07-2000

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 125 946 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
22.08.2001 Bulletin 2001/34

(21) Application number: **00200567.6**

(22) Date of filing: **18.02.2000**

(51) Int Cl.[7]: **C07K 14/47**, C12N 9/16, A61K 38/18, A61K 38/46, C12Q 1/34, A61P 43/00

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(71) Applicant: **Universitair Medisch Centrum Utrecht**
3584 CX Utrecht (NL)

(72) Inventors:
- **Bos, J.L.**
  3584 CX Utrecht (NL)
- **de Rooy, J.**
  3584 CX Utrecht (NL)

(74) Representative: **Ottevangers, Sietse Ulbe et al**
Vereenigde,
Postbus 87930
2508 DH Den Haag (NL)

(54) Cell adhesion therapy through modulation of a Rap family member or of a GEF (guanine nucleotide exchange factor)

(57) The invention relates to the field molecular biology, more specifically to the field of signal transduction in the control of cell adhesion. The invention provides a method for modulating cell adhesion comprising modulating a protein belonging to the Rap family of small GTPases or comprising modulating a guanine nucleotide exchange factor for a protein belonging to the Rap family of small GTPases.

EP 1 125 946 A1

Printed by Jouvè, 75001 PARIS (FR)

Description

[0001] The invention relates to the field of molecular biology, more specifically to the field of signal transduction in the control of cell adhesion.

[0002] Modulation of cell adhesion is a major challenge in medicine. e.g. anti-adhesion therapy may be highly beneficial to prevent among others infiltration of leukocytes in acute inflammations, rheumatoid arthritis, ischaemic reperfusion injury, and auto-immune diseases. Modulation of adhesiveness of tumour cells may inhibit invasiveness and metastatic spread and modulation of adhesiveness of endothelial cells may affect angiogenesis. Compounds central to adhesion are integrins, and the role of integrins in a variety of disease processes where anti-adhesion therapy may be useful have been reviewed extensively. (Ruoslathi, 1999, Hughes and Pfaff, 1998, Kolanus and Seed, 1997 and references therein).

[0003] Integrins are a family of transmembrane, heterodimeric cell adhesion molecules consisting of an alpha and a beta chain that function as receptor for extracellular matrix components. There are a large number of different alpha and beta chains, and the combination determines the specificity of the receptor. Integrins can modulate their adhesive capacity by a complex mechanism including comformational changes to increase affinity and clustering to increase avidity. The mechanism by which the cell regulates integrin function (inside-out signalling) is largely unclear (see below). In addition, when integrins interact with the extracellular matrix, they transduce a signal into the cell (outside-in signalling). A large variety of signalling events occurs after ligand binding, including the activation of FAK, Src, Ras etc.

[0004] To modulate or regulate integrin-mediated adhesion a cell has at least four different ways to control integrin functioning. Firstly, expression of the integrin. Secondly the translocation of integrins from intracellular vesicle to the plasma membrane, Thirdly, the activation of integrins to increase the affinity for the ligand and fourthly the clustering of integrin to increase binding capacity (avidity). Depending on the integrin and the cell types one of more of these mechanisms may predominate. Generally, integrin activation is induced by extracellular stimuli that activate other receptors on the cell surface. For instance, thrombin rapidly activates integrin allbb3 on human platelets (Franke et al., 1997). A large number of studies have been performed trying to elucidate the mechanism of "inside-out" signalling. These studies resulted in the peculiar finding that, although in a variety of primary cells, such as neutrophils and lymphocytes, this process clearly occurs, in most cell lines this regulation is lost. This may be due to the fact that most cell lines are tumor cell lines. Pharmacological studies have revealed that both phosphatidyl inositol-3-kinase (PI3K) and protein kinase C (PKC) are involved in the regulation of most integrins. Furthermore a large variety of proteins that interact with specific alpha or beta-chains have been identified which may be involved in the activation of integrins (Kolanus and Seed, 1997).

[0005] Recently came the finding that small GTPases may be involved in inside-out signalling. The first clear example was the work of Ruoslathi and coworkers (Zhang et al., 1996), showing introduction of an active form of R-ras results in increased adhesion of allbb3. Furthermore, they show that inhibition of R-ras using a dominant negative R-ras mutant results in a decreased adhesion. Furthermore, it was shown that R-ras was regulated in a for GTPases very peculiar manner, i.e. activation of the Eph receptor induces tyrosine phosphorylation of the effector domain of R-ras, resulting in inactivation.

[0006] Also Ras has been implicated in the control in inside-out regulation of integrins (Shibayama et al., 1999). In T-cells dominant negative Ras inhibits TCR-induced adhesion to ICAM, suggesting that Ras plays a positive role in integrin signalling. However, in CHO cells Ras inhibits activation of integrins and the model is proposed that R-ras and Ras operate in distinct pathways, one positive (R-ras) and one negative pathway (Ras) (Hughes et al, 1997). However, little to none is known about how these small GTPases regulate integrin activation (Hughes and Pfaff, 1997).

[0007] The invention provides a method for modulating or regulating cell adhesion comprising modulating a protein belonging to the Rap family of small GTPases, and thus provides targets for drugs that modulate adhesion of cells. These targets are intermediates of the Rap signal transduction pathway, among others guanine nucleotide exchange factors for Rap1. Compounds affecting these targets are beneficial for a large variety of diseases, among others, ischaemic reperfusion injury, auto-immune disease, acute inflammation, rheumatoid arthritis and cancer. The invention provides among others access to and insight in the role of the Rap signalling pathway in the control of integrin-mediated events. These integrin-mediated events control cell adhesion, cell migration, cell proliferation and cell survival.

[0008] The invention also provides the inhibition of the Rap signalling pathway by various means which results in the inhibition of integrin activation. The inhibitors provides by this invention are compounds or drugs that inhibit either Rap or guanine nucleotide exchange factors for Rap.

[0009] The invention also provides a method for modulating cell adhesion comprising modulating a guanine nucleotide exchange factor for a protein belonging to the Rap family of small GTPases, and thus also provides among others access to and insight in the mechanism by which guanine nucleotide exchange factors for Rap are regulated. From our work we have established that a number of these guanine nucleotide exchange factors are regulated by auto-inhibitory domains that needs to be released from inhibition by small molecules. We established the mechanism of activation for Epac, Epac2, members of CalDAG-GEF family and the PDZ-GEF

family of guanine nucleotide exchange factors. Our invention provides that these guanine nucleotide exchange factors are ideal targets for drugs modulating cell adhesion.

[0010] The invention provides a method according to the invention comprising modulating said cell adhesion by modulating integrin-mediated events in said cell adhesion, in that Rap and guanine nucleotide exchange factors for Rap are targets for compounds to modulate cell adhesion through among others integrins, in particular by modulating the auto-inhibitory domain of a guanine nucleotide exchange factor for a protein belonging to the Rap family of small GTPases. Of course, the invention also provides use of a modulator of a protein belonging to the Rap family of small GTPases for the preparation of a medicament for adhesion or anti-adhesion therapy or use of a modulator of a guanine nucleotide exchange factor for a protein belonging to the Rap family of small GTPases for the preparation of a medicament for adhesion or anti-adhesion therapy, in particular wherein said adhesion or anti-adhesion therapy comprises modulating integrin-mediated events, for example wherein said modulator comprises a modulator of the auto-inhibitory domain of a guanine nucleotide exchange factor of a protein belonging to the Rap family of small GTPases.

[0011] Particularly the guanine nucleotide exchange factors for Rap are suitable targets since they are accessible to small molecules. Rap stands for a family of proteins, which consists of the proteins Rap1a, Rap1b, Rap2a and Rap2b. A guanine nucleotide exchange factor for Rap is any protein that elevates the release of GDP from Rap1 by direct physical interaction between the guanine nucleotide exchange factor and Rap. These guanine nucleotide exchange proteins include at least C3G, three members of the Epac family, three members of the CalDAG-GEF family and two members of the PDZ-GEF family.

[0012] A GTPase activating protein for Rap is any protein that increases the hydrolysis rate of GTP when bound to Rap. These GTPases activating proteins include at least RapGAP and RapGAPII and Spa1. An effector of Rap is any protein that binds directly to Rap and functions in mediating signals from Rap to a biological response. A modulator of Rap is any proteins, lipid or natural compound that influences the functioning of the Rap signalling pathway

[0013] The Rap signalling pathway comprises proteins, lipids and natural compounds that constitute a signalling pathway involving Rap.

[0014] The invention also provides a method for identifying a modulator of a protein belonging to the Rap family of small GTPases or of modulator of a guanine nucleotide exchange factor for a protein belonging to the Rap family of small GTPases comprising providing for binding of a nucleotide or nucleotide analogue to a protein belonging to the Rap family of small GTPases, incubating said protein in the presence of a guanine nucleotide exchange factor of a protein belonging to the Rap family of small GTPases and incubating said protein in the presence of a candidate modulator compound and measuring release of said nucleotide or nucleotide analogue from said protein belonging to the Rap family of small GTPases. For example, the invention provides a method for detecting a modulator that activates (activator) using a method wherein activation of said release is measured, e.g. by loading Rap1 with mantGDP, incubating it in the presence of guanine nucleotide exchange factors containing the auto-inhibitory domain, adding test compounds (candidate modulator compounds)and measuring the release of mantGDP spectroscopically. In another example, the invention provides a method for detecting a modulator that inhibits (inhibitor) using a method wherein activation of said release is measured, e.g. by loading Rap1 with mantGDP, incubating it in the presence of guanine nucleotide exchange factors containing the auto-inhibitory domain and in the presence of an (natural) activator, adding test compounds (candidate modulator compounds)and measuring the inhibition of the release of mantGDP spectroscopically.

[0015] In such a way, the invention provides a modulator obtainable by a method according to the invention. Such modulators are for example compounds, which modulate the activity of a guanine nucleotide exchange factor for Rap, Rap, a GTPase activating protein for Rap, an effector for Rap or a modulator of the Rap signalling pathways and which are useful in the modulation of cell adhesion, exemplified by compounds which modulate the functioning of the autoinhibitory domain of a guanine nucleotide exchange factor for Rap1a and Rap1b, such as short peptides, or even large molecules such as antibodies reactive with said autoinhibitory domain, which results in the modulation of integrin-mediated cell adhesion. The invention provides use of such a modulator in a method for modulating cell adhesion or use for the preparation of a medicament, in particular for for adhesion or anti-adhesion therapy, and provides such a medicament. The invention is further explained in the detailed description without limiting the invention thereto

Detailed description

[0016] The small GTPase Rap1, a close relative of Ras, was first described as a revertant of K-ras transformed cells. Since the effector domain of Rap1 is very similar to the effector domain of Ras, the model was proposed that Rap1 interacts with the same effectors as Ras, but inhibit, rather than activate these effectors. This antagonistic effect on Ras signalling is still one of the major models for the function of Rap1 that appear in the literature. However, from our work of the last few years this model is heavily challenged. Using newly developed assays for the activation of Rap1 we observed that Rap1 is rapidly activated by a large variety of stimuli, including stimuli that also activate Ras. Common second messen-

gers, like calcium, diacylglycerol and cAMP, activate a number of GEF for Rap1 directlyresulting in the activation of Rap1. (See Figure 11) Also the downregulation of Rap1 is regulated. For instance, the alpha-subunit of Gi directly associates with and inactivates RapGAP. Importantly, when these stimuli are used to activate Rap1, we did not observe inhibition of the Ras signalling pathway. From these and other results we concluded that Rap1 more likely has a distinct function. We therefore embarked on the identification of this distinct pathway and found that at least one of the pathways in which Rap1 is involved is the regulation of integrin-mediated adhesion.

Example 1

Rap1 mediates inside-out signalling to regulate integrin-mediated cell adhesion

[0017] Integrin-mediated leukocyte adhesion is a critical aspect of leukocyte function that is tightly regulated by diverse stimuli, including chemokines, antigen receptors, and adhesion receptors. How cellular signals from CD31 and other adhesion amplifiers are integrated with those from classical mitogenic stimuli to regulate leukocyte function remains poorly understood. Several reports suggest a role for Ras family GTPases in the control of integrin-mediated adhesion. Ras superfamily GTPases cycle between inactive GDP-bound forms and active GTP-bound forms, and exchange for GTP and hydrolysis of GTP to GDP are catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively (Bos, 1997; Bos 1998). Use of active and dominant-negative mutants of H-Ras and R-Ras has revealed that these enzymes can regulate coupling of betal and beta2 integrin-dependent adhesion to T cell receptor (TCR/CD3), interleukin-3, and chemokine receptor signaling in T lymphocytes and pro-B cell leukemic cell lines (Liu et al., 1999; O' Rourke et al., 1998; Shibayama et al., 1999; Tanaka et al., 1999; Zhang et al., 1996). We have examined whether CD31 stimulates integrin-dependent adhesion via an intracellular signaling pathway, and if so, whether members of the Ras family were involved in this process. We identify the small GTPase Rap1 as a critical mediator of this effect. Importantly, CD31 selectively activated the small Ras-related GTPase Rap1, but not Ras, R-Ras or Rap2. An activated Rap1 mutant stimulated T lymphocyte adhesion to ICAM and VCAM, as did the Rap1 guanine nucleotide exchange factor C3G and a catalytically inactive mutant of RapGAP. Conversely, negative regulators of Rap1 signaling blocked CD31-dependent adhesion. These findings identify a novel important role for Rap1 in regulating ligand-induced cell adhesion and suggest that Rap1 may play a more general role in coordinating adhesion-dependent signals during leukocyte migration and extravasation.

[0018] To address which Ras family members, if any, might participate in CD31 signaling, we precipitated endogenous GTPases from lysates of CD31-stimulated Jurkat with GST fusion proteins of Ras family-binding domains (RBDs) of Raf and RalGDS, which bind with high selectivity and specificty to activated Ras and Rap GTPases, respectively (de Rooij and Bos, 1997; Franke et al., 1997). Immunoblotting of precipitated GTPases provides a qualitative representation of GTPase activation status following cell stimulation, corresponding to quantitative changes detected using classical GDP/GTP-binding ratio techniques. Time-course analyses of Jurkat cells treated with anti-CD31 antibodies or TPA (Figure 1A) surprisingly revealed a rapid activation of the Ras-related GTPase Rap1 by CD31, which was maximal 2-5 minutes post-stimulation and decreased slowly toward basal levels after 20 minutes. In contrast, while both Rap1 and Ras were activated by TPA, CD31-dependent stimulation of Rap1 was highly selective and no activation of Rap2, Ras or R-Ras was noted at any point during this time-course. Both Rap1 and Ras, but not the other GTPases tested, were also activated by TCR stimulation (data not shown). GTP-bound R-Ras was detected in Raf-RBD pull-downs only following longer exposures of films, reflecting the relatively low levels of R-Ras protein expression in Jurkat. R-Ras protein expression was at least 10-fold lower in Jurkat cells as compared to A431 fibroblasts, while Rap1, Rap2, and Ras levels were relatively equivalent (right panels, last two lanes).

[0019] Functional anti-CD31 antibodies against CD31 extracellular domains 1 and 6 (PECAM 1.2, PECAM 1.3 and 2H8), activated Rap1 more potently than non-functional antibodies (WM59, 9G11, GI18) (Figure 1B), correlating with the ability of these antibodies to induce CD31-dependent adhesion (Newton et al., 1997). Additionally, activation of Rap1 by anti-CD31 antibodies was observed in CD31 WT Jurkat transfectants but not CD31 GPI or Y663/686F transfectants (Figure 1C), demonstrating a correlative requirement for the CD31 cytoplasmic tail in the activation of Rap1 and induction of adhesion.

[0020] As CD31 stimulation selectively activated Rap1 and Rap1 activation correlated with CD31-dependent adhesion to ICAM and VCAM, we next addressed whether constitutive activation of Rap1 was sufficient to induce adhesion. Jurkat T cells transiently transfected with a luciferase reporter plasmid and indicated cDNA constructs were allowed to bind to immobilized recombinant ICAM and adhering transfected cells detected by measurement of luciferase activity. T cells expressing constitutively active Rap1 (RapV12) or the Rap1 GEF C3G (Tanaka et al., 1994) displayed approximately 3.5- and 3-fold increases in adhesion to ICAM, respectively (Figure 2A). C3G-induced spreading and adhesion on fibronectin has been previously observed in 32D cells, although involvement of Rap1 was not examined and Ras, R-Ras, or other GTPases were implicated (Arai et al., 1999). Expression of either the active

form of Ras (RasV12) or R-Ras (R-RasV38) resulted in a lower but detectable induction of adhesion (1.5-2 fold). However, RasV12-stimulated adhesion was not statistically significant, RasV12 stimulated adhesion in only two of five experiments, and RapV12-induced adhesion was always higher than that induced by RasV12 and R-RasV38, even though the latter two constructs were expressed at significantly higher levels than RapV12 (Figure 2A, left inset). Introduction of a RapGAP (Rubinfeld et al., 1991) mutant (RapGAP-LIG), containing a substitution of amino acids 284-286 in the RapGAP arginine finger critical for RapGAP catalytic activity (RKR, mutated to LIG) which unlike wild-type RapGAP displays no GAP activity toward Rap1 (Figure 2B), increased the number of ICAM-bound cells to a similar level as observed with RapV12 and C3G. RapGAP-LIG may perturb Rap1 signaling by displacing endogenous RapGAP from a subcellular localization required for down-regulating Rap1 or may constitutively associate with basally activated Rap1 and serve as an effector protein linking Rap1 to an as yet unidentified target.

[0021] We next examined if inhibition of Rap1 signaling would abolish CD31-dependent adhesion to ICAM. We therefore introduced a putative dominant negative mutant of Rap1, RapN17, into cells. RapN17 did not affect basal adhesion of Jurkat to ICAM, but completely abolished CD31-dependent increases in adhesion (Figure 2C). Although the RapN17 mutation was modeled on the corresponding dominant-negative mutation in H-Ras (Medema et al., 1991), RapN17 is not considered to function as a strict dominant-negative protein. Whereas RasN17 blocks Ras signalling by sequestering Ras GEFs, RapN17 fails to bind to Rap1 GEFs in *vitro* (van den Berghe et al., 1997), and fails to inhibit EGF-dependent activation of cotransfected wild-type Rap1 in COS-7 cells (R.M.F.W. and J.L.B., unpublished observation).

[0022] Because of uncertainties in the mechanism by which RapN17 might block CD31-stimulated adhesion, we sought independent confirmation that Rap1 was required for CD31-induced adhesion of Jurkat to ICAM. Transient expression of the Rap1-specific GAP RapGAP resulted in a strong reduction of basal adhesion to ICAM, and significantly reduced CD31-dependent adhesion (Figure 2C). This result is compatible with the notion that a Rap1GAP should decrease levels of GTP-bound Rap1 (see Figure 2B) but should still allow ligand-induced activation of Rap1. Finally, we used the Rap1 binding domain of RalGDS, RBD, which binds with high affinity and specificity to GTP-bound Rap1 *in vitro* (Franke et al., 1997). This fragment is hypothesized to block effector binding to Rap1. Expression of RBD significantly abolished both basal and CD31-induced adhesion to ICAM (Figure 2C). Thus, using three independent strategies for interfering with Rap1 signaling, overexpression of inactive Rap1, Rap1-specific RapGAPs, and an isolated RBD of a Rap-binding protein, these experiments demonstrate a critical role for Rap1 in mediating CD31-induced adhesion to ICAM.

[0023] To address whether Rap1 selectively regulated b2 integrins (LFA-1) on Jurkat, or was also coupled to the regulation of b1integrins (VLA-4), we examined the effects of activating and inactivating mutants of Rap signaling on Jurkat adhesion to VCAM. Stimulation of CD31 resulted in a smaller induction of adhesion to VCAM (1.5-2 fold increase) (Figure 3A) than observed to ICAM, in part due to the higher basal level of adhesion observed on VCAM (typically 30-50%, compared to 10-20% on ICAM). A similar increase was also observed in cells expressing RapV12, C3G, and mutant RapGAP-LIG, but not RasV12 or R-RasV38. Conversely, RapN17, RapGAP, and a second GAP for Rap1, Spa1 (Kurachi et al., 1997), significantly reduced basal Jurkat adhesion to VCAM, and strongly blocked CD31-dependent adhesion (Figure 3B).

[0024] It has been previously reported that Ras regulates TCR-stimulated lymphocyte adhesion to ICAM (O'Rourke et al., 1998; Tanaka et al., 1999). As we failed to detect Ras activation by CD31, and RasV12 did not induce a clear increase in adhesion to ICAM or VCAM, it is unlikely that Ras mediates the main signalling pathway from CD31 stimulation to integrin-mediated adhesion. However, overexpression of RasN17 did inhibit CD31-dependent adhesion to VCAM (data not shown), suggesting that Ras might also contribute to adhesion responses.

[0025] Integrin-dependent adhesion can be regulated by changes in integrin surface expression, integrin surface distribution (avidity), or induction of conformational changes which increase integrin ligand affinity (Stewart and Hogg, 1996). To investigate at which level Rap1 might influence cell adhesion, we first examined if Rap signalling affected LFA-1 expression levels. Jurkat cells were cotransfected with EGFP as a reporter marker, along with indicated Rap1 signalling pathway cDNA constructs. Staining of transfected cells with anti-b2 integrin antibody revealed that overexpression of RapV12, RapN17, RBD, RapGAP or RapGAP LIG failed to influence surface expression levels of LFA-1 (Figure 4A). CD31 expression levels were also unaffected (data not shown). Similarly, RapV12, RapGAP LIG, RapGAP and RapN17 failed to induce LFA-1 surface clustering, as detected with the NKI-L16 antibody (data not shown), although active RasV12 induced clustering of LFA-1, as previously reported (Tanaka et al., 1999).

[0026] The anti-aL integrin antibody mAB 24 recognizes a conformation-dependent ligand-induced epitope on LFA-1, and induction of the mAB 24 epitope correlates with increased LFA-1 affinity for ICAM (Dransfield and Hogg, 1989). TCR and TPA-induced mAB 24 epitope expression have previously been found to be dependent on the presence of ICAM, suggesting that signals generated by TCR and TPA facilitate weak basal interactions between LFA-1 and ICAM, stabilizing LFA-1 in a high-affinity conformation (Cabanas and Hobb, 1993). This conformational change can also be

induced by LFA-1 exposure to Mn^{2+}, independently of "inside-out" signalling. We found that anti-CD31 stimulation of Jurkat, or Jurkat cells transfected with GFP alone, resulted in an approximate 1.5-fold increase in mAB 24 expression (data not shown and Figure 4B), while a 2.25-2.5-fold increase was observed in Mn^{2+}-treated cells. Incubation of cells with mAB 24 at 4°C abolished CD31 and Mn^{2+} -induced mAB 24 expression to background levels (data not shown), consistent with the temperature-sensitivity of the mAB 24 epitope (Dransfield and Hogg, 1989). Although RapV12 expression had no statistically significant effect on basal or induced mAB 24 epitope expression, RapN17 and RBD abolished induction of the mAB 24 epitope by CD31 and Mn^{2+}. Conversely, RapGAP LIG enhanced CD31- and Mn^{2+}- induced expression of the mAB 24 epitope. RapN17 also inhibited the increase in the percentage of mAB 24 -reactive cells observed following CD31 or Mn^{2+} treatment (Figure 4C). Thus, our studies suggest that while Rap does not directly stimulate activation of LFA-1, signalling via Rap1, or basal activation of Rap1, appears to be a requisite factor for stabilization of the active conformation of LFA-1. Moreover, our finding that RapN17 blocks direct conformational changes induced by extracellular Mn^{2+} may suggest that inactive Rap may act to lock LFA-1 in an inactive conformation. Consistent with this model, overexpression of RapN17, RapGAP or RalGDS RBD significantly blocked Mn^{2+}-induced adhesion of Jurkat cells to immobilized ICAM, while RapV12 augmented the induced cell adhesion (Figure 4D).

[0027] Our report is the first example of requisite involvement of Rap1 in coupling cell surface receptor stimulation to integrin-mediated adhesion. Previously it was shown that Rap1 is activated after stimulation of a large variety of cell surface receptors, including B and T cell antigen receptors, chemokine and cytokine receptors (including fMLP, PAF, and GM-CSF), and receptors for platelet agonists (thrombin, thromboxane A2 and ADP) (reviewed in Bos, 1997). Many of these receptors have been implicated in the control of integrin-mediated adhesion.

Experimental procedures example 1

[0028] Hemagglutinin (HA)-tagged Rap1 (Rap), RapV12, and H-RasV12 in the mammalian expression vector pMT2HA, pRSV, and pRSV-RapN17 have been previously described (Zwartkruis et al., 1998). pMT2HA-R-RasV38 was generated by subcloning a full length *Nco I* (Klenow-filled) /*Xho* I fragment of R-RasV38 into *Not I* (mung bean nuclease-digested) /*Xho* I-digested pMT2HA. A *Sal I*/*Not I* fragment encoding amino acids 200-297 of human RalGDS was amplified by PCR to generate pMT2HA-RalGDS-RBD. pMT2-HA-RapGAP was generated by subcloning full-length RapGAP (provided by Dr. Paul Polakis) as a *Sal I/ Bgl II* fragment into pMT2HA. Catalytically inactive RapGAP LIG was generated by mutating RapGAP amino acids 284-286 (RKR) to LIG using the Stratagene Quick-site-directed mutagenesis kit. All PCR-generated sequences were checked by DNA sequencing. pCAGGS-C3G and pSR-His-tagged Spa1 were provided by Drs. Michiyuki Matsuda (NIH, Tokyo) and Masakazu Hattori (Kyoto University), respectively.

[0029] Jurkat T cell lines were maintained as previously described (Reedquist and Bos, 1998). The Jurkat line JHM1 2.2 was provided by Dr. Doreen Cantrell, with kind permission of Dr. Art Weiss. Jurkat cells used to generate CD31 WT, CD31 GPI, and Y663/686F stable transfectants were provided by Dr. D. Samson (University of Bath). CD31-positive and -negative variants were established by six rounds of MACs-sorting of parental Jurkat with anti-CD31 antibody 10B9. The negative variant was transfected with CD31 WT, CD31 GPI, or CD31 Y663/686F cDNA in pCDNA3 (Jackson et al., 1997; Newton et al., 1997) and stable polyclonal lines established by selection in G418 (1 mg/ml) and FACS sorting. Jurkat cells were transientally transfected by electroporation with 35 μg plasmid DNA. Jurkat cells (1.2×10^7 cells/ml in 0.4 ml complete media) were pulsed at 250 V and 960 μF with 5μg TK-luciferase plasmid DNA, construct plasmids as indicated in figure legends, and added vector plasmid to keep DNA amounts constant. 24 hours post-transfection, cells were transferred to serum-free media and used 42-48 hours post-transfection for adhesion assays. Subconfluent A14 and COS-7 cells were transfected by calcium phosphate precipitation as previously described (Zwartkruis et al., 1998).

[0030] Anti-CD3 antibody T3b was kindly provided by Dr. Hergen Spits (Netherlands Cancer Institute, Amsterdam). Anti-integrin b2 antibody L15, activating anti-integrin μ1 antibody TS2/16 and activating anti-integrin b2 KIM185 have been previously described (Andrew et al., 1993; Van de Wiel-van Kemenade et al., 1992). mAB 24, recognizing a ligand-induced epitope of μL, was kindly provided by Dr. Nancy Hogg (ICRF, London). Anti-CD31 antibodies PECAM 1.2 and PECAM 1.3 were generous gifts from Dr. Peter Newman. Anti-CD31 antibodies 2H8, WM59, GI18 and 9G11 have been previously described (Newton et al., 1997; Yan et al., 1995). Other monoclonal antibodies used in these studies were anti-Rap1, RalA and Ras antibodies (from Transduction Laboratories). Rabbit polyclonal anti- Rap2 and R-Ras antibodies were from Santa Cruz.

[0031] Purification of GST-RBD fusion proteins and their use in detecting activated Rap1, Rap2, Ras, and R-Ras by precipitation, SDS-PAGE and immunoblotting have been previously described (de Rooij and Bos, 1997; Franke et al., 1997; Reedquist and Bos, 1998). Detection of bound radiolabelled GDP and GTP to Rap1 was also as previously described (Zwartkruis et al., 1998).

[0032] For adhesion assays, transiently transfected Jurkat cells were harvested, washed and resuspended in TSM media (20 mM Tris, pH 8, 150 mM NaCl, 1 mM

$CaCl_2$, 2 mM $MgCl_2$). 96-well Costar Maxisorp plates were coated overnight at 4°C with goat anti-human IgG antibodies (Jackson, 4 µg/ml in TSM), washed, blocked 30 minutes at 37°C with 1% bovine serum albumin (BSA)/TSM, followed by incubation 1 hour at 37°C with 50 ng/ml recombinant ICAM-1/ or VCAM-1/ human IgG Fc fusion proteins. 50 µl cell suspension was mixed with 50 µl TSM or TSM with stimuli indicated in Figure Legends. Cells were allowed to adhere for 30-60 minutes, and nonadherent cells removed with warm 0.5%BSA/ TSM. Adherent cells were lysed and subjected to luciferase assays as previously described (Medema et al., 1992). Expression of transfected constructs was confirmed by immunoblotting of total cell lysates. Cells bound were calculated as in Figure Legends, and were corrected for transfection efficiency and non-specific effects of constructs by measuring luciferase activity of total input cells. Comparison of effects of cDNA construct overexpression on adhesion were made by paired t test or unpaired (Student's) t test as appropriate. Fluorescent labelling of stably transfected cells with 2'7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein acetoxymethyl ester (Molecular Probes) and measurement of adherent cells with a Fluoroscan Ascent fluorescent plate reader (Lab Systems) has been previously described (Newton et al., 1997).

[0033] Flow cytometric analysis of transfected cells was performed by cotransfecting Jurkat JHMI cells with Img pCMV-EGFP C1 plasmid (Promega) and the indicated cDNA constructs. Following overnight serum-starvation, cells were equilibrated in 0.5% BSA/1mM $CaCl_2$ /PBS (FACS Buffer) and left unstimulated, or stimulated for 30 minutes with plate-immobilized anti-CD31 antibody 2H8 (10 mg/ml). Cells were harvested, resuspended in FACS Buffer containing primary antibodies (10 mg/ml) and incubated for 30 minutes on ice, or 37°C for mAB 24 epitope expression. For mAB 24 staining, additional sets of transfected cells were coincubated with 400 mM $MnCl_2$ during primary antibody staining. Cells were washed with FACS Buffer and stained with secondary rabbit anti-mouse RPE-Cy5-conjugated antibodies (Dako). Fluorescence intensity of EGFP-transfected cells was determined using a FACsCaliber flow cytometer and CellQuest software (both from Becton Dickinson).

Example 2

[0034] Guanine nucleotide exchange factors for Rap are regulated by autoinhibitory domains that respond to intracellualr second messengers.

[0035] A number of guanine-nucleotide exchange factors (GEFs) have been identified which mediate the activation of Rap1. The first described Rap1GEF, C3G, is found in a complex with the proto-oncogene product c-Crk and may activate Rap1, as a consequence of complex formation and translocation, induced by receptor tyrosine kinase signaling. CalDAG-GEFI has calcium-binding EF-hands and a domain that resembles C1-type DAG-binding domains and may explain the activation of Rap1 by these two second messengers. Recently, another type of GEF for Rap1, called PDZ-GEF1 or nRapGEP or RaGEF was described. This GEF contains, in addition to the catalytic region, a Ras-binding domain (RED) that may interact directly with Ras and Rap1 in vitro, a PDZ domain that drives membrane association and a domain that is related to cAMP-binding domains (RCBD), but does not bind cAMP. Another RapGEF is Epac (exchange protein directly activated by cAMP), because this GEF represents a novel target for cAMP, independent from the classical target protein Protein Kinase A (PKA) [de Rooij, 1998; Kawasaki, 1998]

[0036] Here we have studied the regulation and function of the different Epac family members in more detail. First, we observe that all three members activate in addition to Rap1 also the close relative Rap2. Secondly, we identified an additional putative cAMP-binding site in Epac2, located N-terminal to the DEP domain. Thirdly, mutant analysis revealed that the cAMP-binding domains proximal to the catalytic domains in Epac1 and Epac2 (the B-sites) function as inhibitors of the GEF domains in the absence of cAMP. Fourthly we show that CalDAG-GEF is also regulated by an auto-inhibitory domain which is modelated by calcium binding. Finally we show that PDZ-GEF can be regulated by a small molecule present in Jurkat cell lysate

[0037] Epac family members activate both Rap1 and Rap2

[0038] Currently, the Epac family of GEFs consists of three members, Epac1 and Epac2 that are regulated by cAMP and Repac (Related to Epac), a member, which lacks any apparent regulatory sequences (Figure 1A). Previously, it was shown that these GEFs activate Rap1 *in vivo* as well as *in vitro*, but not the closely related GTPases Ras, R-ras or Ral. We have extended these experiments and found that all three GEFs can directly activate Rap2 as well (Figure 1B). Equal amounts (approximately 100nM) of GST-fusions of the catalytic domains (Figure 1A) were incubated with fluorescent mantGDP loaded Rap1 or Rap2 (100nM) in the presence of excess unlabelled GDP and exchange of guanine-nucleotides was followed in realtime as a decrease in fluorescence. To compare activity of the different GEFs towards Rap1 and Rap2, single exponential curves were fit from which the exchange reaction rates were calculated. These rates were compared to the intrinsic exchange reaction rates of the GTPases measured in the same experiment. From these calculations a fold induction of guanine-nucleotide exchange on Rap1 and Rap2 was derived, which is depicted in Figure 1C. Epac1 activated Rap2 five times more efficiently than Rap1, whereas Epac2 and Repac activated Rap2 three fold less efficiently (Figure 1B). Activation of Rap2 is not a common feature of all Rap1GEFs, since C3G and CalDAG-GEFI (Figure 1B) did not exhibit catalytic activity towards

Rap2 *in vitro*.

[0039] To validate the results obtained *in vitro*, we investigated whether the Epac family members also activate Rap1 and Rap2 *in vivo*. Cells were transfected with Epac cDNAs together with either Rap1 or Rap2 and stimulated with forskolin and IBMX to raise the level of cAMP. Activation of Rap1 was measured using the previously described activation-specific probe assay in which Rap1GTP is specifically precipitated with the Rap1 binding domain of Ra1GDS. As shown in Figure 1C, all three GEFs activate Rap1. The activation by Epac1 and Epac2 is enhanced by forskolin treatment.

[0040] The activation specific probe assay is less suitable for measuring activation of Rap2, due to the high basal level of Rap2GTP in cells. Therefore, we incubated the cells with 32P-labelled orthophosphate followed by precipitation of Rap2, and separation of bound GDP and GTP. We observed that all three GEFs activate Rap2 *in vivo* (Figure 1D). The activation of Rap2 by Epac1 and Epac2 is enhanced by forskolin treatment. From these results we conclude that all three members of the Epac family, activate both Rap1 and Rap2.

[0041] Epac2 has a second, low-affinity binding site for cAMP.

[0042] In the completed sequence of the C. elegans genome, only one Epac related gene could be found. This gene encodes a protein that has a putative second cAMP-binding domain at the N-terminus, apart from the reported cAMP-binding domain proximal to the catalytic region. This domain is not present in Epac1, but in Epac2 a similar cAMP-binding site is present (Figure 1A). As judged from primary sequences, this site is similar to the genuine cAMP-binding sites of Epac1, Epac2 and PKA, but distinct from the RCBD domain in PDZ-GEF, a RapGEF which does not respond to cAMP (Figure 2A). We named the N-terminal cAMP-binding domain present in Epac2 and C. elegans Epac the A-site and we named the cAMP-binding domain proximal to the catalytic domains, which is present in Epac1, Epac2 and C.elegans Epac the B-site (Figure 2A). To compare these different sites, we analyzed purified domains (Figure 2A) for in vitro binding to cAMP by isothermal titration calorimetry (ITC). We found that the A-site of Epac2 binds cAMP with an apparent affinity of 87μM, whereas the B-site has an affinity of 1.2 μM, which is comparable to the affinity of 4μM, observed for the cAMP-binding domain of Epac1 (Figure 2B). Apparently, the A-site has a much lower affinity for cAMP as compared to the B-site.

[0043] In the regulatory subunits of PKA two cAMP-binding domains are present that cooperatively bind to cAMP, meaning that the binding of cAMP to one site influences the affinity of the second site for cAMP. To investigate whether also site A and B in Epac2 may act cooperatively, we measured cAMP-binding affinity to the complete regulatory region of Epac2. Best-fit analysis revealed two binding sites with Kd's of 0.5 and 76 μM, (Figure 2C), which are in the same range as the affinities of the isolated domains. As a final control a mixture of the separately purified A and B cAMP-binding sites of Epac2, in which no cooperativity can occur, was analyzed in the same assay. This yielded exactly the same result as the titration of the complete regulatory domain of Epac2 (Figure 2C lower panel). The data from these measurements are summarized in Figure 2D. We conclude that no cooperativity occurs in binding of cAMP to the regulatory domain of Epac2 and that also in the full length Epac2 protein, the B-site has a much higher affinity for cAMP that the A-site.

[0044] Isolated B-sites inhibit the exchange activity of Epac catalytic domains.

[0045] To investigate the role of the different N-terminal domains in the regulation of Epac1 and Epac2 activity by cAMP, we made several deletion constructs (Figure 3A). As shown in Figure 3B, mutant Epac1 (Epac1-DDEP) and Epac2 (Epac2-DDEP) proteins containing, next to the catalytic domain, only the B-site cAMP-binding domain respond to cAMP *in vitro*, like full-length Epac1. However, proteins that lack the B-sites as well as the other N-terminal domains are constitutively active (see Figure 1B). This implies that the B-sites serve as auto-inhibitory domains. Next we investigated whether a direct covalent linkage between the catalytic domain and the B-site is essential for this regulation, or whether they can function as separate domains. We therefore isolated the regulatory domains of both Epac1 and Epac2 and incubated them with the corresponding catalytic domains. As shown in Figure 3C, both regulatory domains completely inhibit the catalytic activity of the corresponding GEF domains, showing that they can form a stable complex that prevents GEF activity. Addition of cAMP abolishes the inhibitory effect. To dissect the role of the two cAMP-binding sites in the regulatory domain of Epac2, purified domains of the A- and the B-site of Epac2 were incubated with the catalytic domain of Epac2. Only the B-site and not the A-site (even at high concentration) inhibits the catalytic domain of Epac2 (Figure 3D). The use of cAMP-binding domain constructs containing also the DEP domain did not alter the ability of the A-site or B-site to inhibit the catalytic activity (data not shown).

[0046] The mechanism of Epac regulation is conserved in a subset of RapGEFs.

[0047] To investigate whether the cAMP-binding domain of Epac1 can regulate only the catalytic domain of Epac1, we incubated the regulatory domain of Epac1 with the catalytic domains of the other RapGEFs (Figure 4A). As shown in Figure 4B, Epac1-RD inhibited the catalytic activity of both Epac2 and Repac. Interestingly, also the catalytic activity of PDZ-GEF, was inhibited. In contrast, the GEF activity of the catalytic domains of C3G and CalDAG-GEFI was not inhibited. From these results we conclude that the isolated regulatory domain of Epac1, can act as an inhibiting structure for a specific subset of RapGEFs. This indicates that this mechanism of regulation is conserved between Epac and PDZ-GEF.

In PDZ-GEF a structure related to cAMP-binding domains (RCBD) is present that probably plays a similar role as the B-sites of Epacs in the regulation of GEF activity. Furthermore, this property is specific for certain cAMP-binding domains only, because neither the A-site of Epac2 (Figure 3C), nor a PKA construct containing both its cAMP-binding domains (Figure 4B) did affect the activity of the catalytic domain of Epac2. Thus we conclude that a specific sequence or structure in the B-sites of Epacs enables these domains to form an inhibitory interaction with the catalytic domains of a subset of RapGEFs.

[0048] Interestingly, Epac1-RD was able also to inhibit the catalytic domains of Epac2, Repac and PDZ-GEF. This indicates that the mechanism by which the B-site interacts with the catalytic domain is rather conserved. It is obvious that the presence of both domains in one protein facilitates this mode of regulation, but it could be hypothesized that originally, in early evolution, the two domains were expressed as separate proteins. This question is particularly interesting with respect to Repac, which lacks any intrinsic regulatory domain. It is well possible that a separate regulatory domain, which has not yet been identified, regulates this GEF. Alternatively, Repac is a constitutively active GEF, which is responsible for basal levels of Rap1GTP and Rap2GTP.

[0049] We therefore investigated whether CalDAG-GEF was regulated by an auto-inhibitory domain. To that end full length CalDAG-GEF 1 and a CalDAG-Gef mutants lacking the regulatory domain was compared for in vitro activation of Rap1. As shown in Figure the full length construct was significantly less effeicient in activating Rap1. However, when calcium was added to the incubation full length CalDAG-GEF became as efficient as the mutant. From these results we conclude that also for CalDAG-GEF the regulatory domain is an auto-inhibitory domain that is modulated by the binding of a second messenger.

[0050] Finally we investigated PDZ-GEF. We had shown previously that deletion of the N-terminal domain related to the cAMP-binding site in Epac (RCBD) results in a more active guanine nucleotide exchange factor for Rap. This suggested to us that perhaps the RCBD is the binding site for a second messenger which regulated PDZ-GEF. Since this domain did not bind to cAMP, we tried lysates from Jurkat cells separated by size exclusion chromatography. Jurkat cells stimulated with serum clearly contain a compound able to activate full length PDZ-GEF. This shows also PDZ-GEF is regulated like Epacs and CalDAG-GEFs, that is by second messengers, which modulate an auto-inhibitory domain.

Experimental procedures example 2

[0051] Constructs used for expression of GEFs and small GTPases in mammalian cells are cloned in the PMT2-SM-HA eukaryotic expression vector. Epac1 constructs are derived from human cDNA and Epac2 constructs are derived from murine cDNA. HA-Epac1-DDEP contains Epac1 lacking amino acids 71-140, which span the DEP domain. For purification of Glutathione S-transferase (GST) -fusion constructs all cDNAs were cloned in pGEX bacterial expression vectors. The catalytic domain of Epac1 contains amino acids 324-881, the regulatory domain of Epac1 (Epac-RD) contains amino acids 2-329, the cAMP-binding domain of Epac1 contains amino acids 149-318 and Epac1-DDEP contains amino acids 149-881. The catalytic domain of Epac2 contains amino acids 460-993, the regulatory domain contains amino acids 1-463, the cAMP-binding site A contains amino acids 1-160, the B-site contains amino acids 280-463 and Epac2-DDEP contains amino acids 280-993. The GST fusion construct of Repac contains amino acids 2-580. The catalytic domain CalDAG-GEFI contains amino acids 3-422. The catalytically active PDZ-GEF1 construct contains amino acids 251-1001. The GST-PKA fusion construct used contained the R1a-subunit of Bovine PKA, lacking amino acids 1-91. Protein production was induced in BL-21 bacteria using 100 nM IPTG for 20 h at room temperature. After protein production, bacteria were pelleted and lyzed in ice-cold PBS containing 1% Triton-X100 and protease inhibitors. The lysate was sonicated three times for 10 seconds and centrifuged at 10,000 x g to remove insoluble material. GST-fusion proteins we purified from the cleared lysate by batch-wise incubation with glutathione-agarose beads (Sigma), eluted from the beads in buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 10% glycerol and 10mM glutathione and dialyzed for 20 hours in the same buffer without glutathione. When indicated, proteins were cleaved from the GST-tag by incubation with thrombin and purified by gelfiltration. Small GTPases used for in vitro experiments were described elsewhere.

In vivo activation of Rap.

[0052] Cells were transfected with HA-tagged Rap1A or Rap2A and serum starved for 20 hours prior to the activation experiments. Cells were stimulated with forskolin (20 mM) and isobutylmethylxanthine (IBMX) (1 mM) for 10 min. The GTP-bound form of Rap1 was specifically isolated using GST-RalGDS as an activation specific probe as described. Detection on westernblot was by 12CA5 monoclonal antibodies directed against the HA-tag. In vivo labeling experiment for Rap2 were performed as well. Briefly, serum-starved cells were labeled with 32P-orthophosphate for 5 hours. Rap2 was precipitated using 12CA5 antibodies, nucleotides were eluted and separated on PEI-cellulose F thin layer chromatography (TLC) plates. Labeled nucleotides were visualized using a phospho-imager and GTP/GDP ratios were calculated using the program ImageQuant.

In vitro activation of small GTPases

[0053] Briefly, 100 nM of purified GTPase, loaded with fluorescently labeled 2',3'-bis (O) -N-methylanthraanoloyl-guanosinediphosphate(mantGDP), was incubated, in the presence of excess unlabelled GDP, with 50 nM of purified GEF unless indicated differently. Release of mGDP was measured in realtime as a decrease in fluorescence. To calculate reaction rates, single exponential functions were fit using the program Grafit3.0 (Eritacus).

Figure legends

[0054] Figure 1. CD31 selectively activates Rap1 but not other Ras family GTPases. A, Jurkat JHMI 2.2 cells were unstimulated (-) or stimulated with anti-CD31 antibody PECAM 1.3 (10mg/ml) for the indicated times or with TPA (100 ng/ml, 5 minutes), lysed and GTP-bound GTPases precipitated with immobilized GST fusion proteins of RalGDS-RBD (for Rap1 and Rap2) or Raf-RBD (for Ras and R-Ras). Bound GTPases were resolved on SDS-PAGE, transferred to PVDF membrane, and detected by immunoblotting with antibodies against the indicated proteins, followed by enhanced chemiuminescence (ECL). B, Antibodies against CD31 extracellular domains regulating cell adhesion activate Rap1. JHMI cells were activated for 2 minutes with medium (-), anti-CD3 antibody T3b (1:200) or the indicated anti-CD31 antibodies (10 µg/ml), and Rap1 activation detected as in A. Fold activation of Rap1 compared to untreated cells was obtained by quantitating pixels from representative blots using Adobe Photoshop software. C, CD31-dependent activation of Rap1 requires CD31 cytoplasmic tail. CD31 WT, CD31 GPI, and Y663/686F Jurkat cell lines were stimulated for two minutes with medium (-) or 10 µg/ml PECAM 1.3 antibody and activated Rap1 detected as in A. Results shown in each panel are representative of three independent experiments.

[0055] Figure 2. Rap1 regulates Jurkat adhesion via LFA-1 (b2) Integrin. A, Activation of Rap1 induces Jurkat adhesion to ICAM. Jurkat JHMI cells were transiently transfected with 5µg PG3- TK-luciferase reporter plasmid and empty vector (control) or pMT2-HA- RapV12, H-RasV12, R-RasV38 (each 10 µg), RapGAP LIG (20 µg) or pCAGGS-C3G (10 µg). Cells were allowed to adhere for 1 hour on immobilized ICAM, washed, lysed, and adherent cells quantitated by luciferase assay. Bars represent the average mean binding and standard error % cells bound from 2-6 independent experiments (indicated above bars) performed in triplicate or quadruplicate, as calculated in Figure 1. Typically, 10-20% of control unstimulated cells adhered to ICAM. ** denotes $p <$ 0.01, * $p < 0.05$, and § $p < 0.1$ compared with control cells by paired *t* test. Expression levels of transfected GTPases and RapGAP-LIG from a representative adhesion assay were examined by immunoblotting lysates with anti-HA epitope antibody 12CA5 as in Figure 1A (insets). Blots shown in insets are cut from a single film exposure of one immunoblot. B, RapGAP-LIG mutant fails to stimulate GTP-hydrolyis on Rap1. A14 cells were transfected with vector alone, or HA-Rap1 alone or in combination with increasing amounts (0.3, 1, 2.5, and 5 µg) of HA-tagged RapGAP or RapGAP-LIG, as indicated above top panel. Active HA-Rap1 was precipitated from cell lysates with RalGDS-RBD and detected by 12CA5 immunoblotting as in Figure 2 (top panel). Transfection of HA-Rap1 (middle panel) and RapGAP constructs (lower panel) was monitored by immunoblotting of total cell lysates with anti-HA antibodies. C, Rap1 signaling is required for CD31-dependent adhesion to ICAM. WT CD31 cells transfected with pG3-TK-luciferase reporter plasmid and 30 µg of empty vector (control) or the indicated DNA construct were allowed to adhere to immobilized ICAM as in (a) following 30 minutes stimulation with medium or 10 µg/ml anti-CD31 antibody PECAM 1.3 cross-linked with goat anti-mouse antibodies. Cells bound were quantitated using luciferase assay and %cells bound cis indicated Shown is a representative experiment performed in quadruplicate. *p* values are indicated as in A and represent transfected cell populations compared to control unstimulated cells (medium) or CD31-stimulated control cells.

[0056] Figure 3. Rap1 mediates CD31-dependent adhesion via b1 (VLA-4) integrins. A, Activation of Rap1 is sufficient to induce adhesion to VCAM. CD31-positive variant Jurkat cells were transfected with 5 µg pG3-TK-luciferase reporter plasmid and empty vector (control), HA-tagged RapV12, H-RasV12, R-RasV38 (10 µg each), C3G (10 µg each) or RapGAP-LIG (20 mg) and allowed to adhere to immobilized VCAM (50 ng/ml). Adherent cells and %cells bound from five (RapV12), three (C3G) or two (RasV12, R-RasV38, and RapGAP LIG) independent experiments performed in triplicate or quadruplicate were quantitated. 30-50% specific adhesion of control cells to VCAM was observed in the presence of medium alone. Cells transfected with RapV12 (** $p < 0.01$) and C3G (* $p < 0.05$), but not RasV12 or R-RasV38 bound at significantly higher levels than control cells. For RapGAP LIG, p was < 0.1003. B, Rap1 signaling is required for CD31-dependent adhesion to VCAM. CD31 WT cells were transfected with 5 mg pG3-TK-luciferase and 30 µg empty vector (control), HA-tagged RapN17, RapGAP, RalGDS-RBD or His-tagged Spa1 were allowed to adhere to VCAM as in A in the absence (control, top panel) or presence of cross-linked anti-CD31 antibody PECAM 1.3 (top panel). Paired t tests of transfected cells revealed that cells transfected with RapGAP (* $p < 0.05$), Spa1 and RED (§ $p < 0.1$) had significantly lower adhesion than control unstimulated cells, and that RapN17, RapGAP ($p < 0.05$), Spa1 and RED ($p < 0.1$) significantly blocked CD31-dependent adhesion.

[0057] Figure 4. Rap signalling regulates ligand-induced conformational changes in LFA-1. A. Expression levels of LFA-1 b2 integrin on transfected cells. Jurkat

JHMI cells were transiently transfected with 1 µg pCMV-EGFP C1 reporter plasmid along with empty vector (GFP) or the indicated cDNA constructs. Cells were sequentially stained with anti-b2 integrin antibody L15 and rabbit anti-mouse Ig-RPE-Cy5 conjugate, and GFP-expressing cells analysed by flow cytometry. Values are presented mean fluorescent intensity (arbitrary units) normalized to 100% for cells expressing GFP alone. B Rap signalling is required for CD31 and Mn^{2+} - induced conformational changes in LFA-1. JHMI cells were transfected as in A and left unstimulated (control) or stimulated 30 minutes with anti-CD31 2H8 antibody (CD31), followed by staining with the ligand-induced binding anti-LFA-1 antibody mAB 24, as in Materials and Methods. Alternatively, unstimulated cells were coincubated with mAB 24 and 400 µM $MnCl_2$ (Mn). mAB 24 expression was assessed by flow cytometry, and expression levels normalized to 1 for unstimulated GFP-alone cells in each experiment. Data represent the means and standard errors of representative experiments (GFP, n=4; RapV12 and RapN17, n=3; RapGAP LIG and RBD, n=2). Statistically significant differences between control and stimulated cells, and between stimulated transfected cells are noted (*, $p < 0.01$; **, $p < 0.05$; §, $p < 0.1$). C RapN17 blocks mAB 24 induction. Experiment was performed as in B but data represents the % of mAB 24-expressing cells. D Rap signalling regulates Mn^{2+}-induced adhesion to ICAM. Jurkat cells were transfected with luciferase reporter plasmid and indicated constructs as in Figure 3 and allowed to adhere to ICAM in absence or presence of 4 mM $MnCl_2$, and % specific adhesion calculated as in Figure 2, and normalized to 100 for mock-transfected stimulated cells. Data represent the average mean and error of 2-4 experiments with each construct performed in quadruplicate, and statistically significant differences are indicated (*, $p < 0.01$; **, $p < 0.05$).

[0058] Figure 5. Epac family members activate both Rap1 and Rap2. a; Bacterially expressed GST-fusion proteins containing the catalytic domains of Epac1 (E1), Epac2 (E2), Repac (Re) and CalDAG-GEFI (CD) were purified using glutathione agarose beads, separated by SDS-polyacrylamide gel electrophoresis and stained by coomassie. b; Purified catalytic domains were incubated at approximately 100 nM with purified Rap1A or Rap2A proteins (100 nM) loaded with mantGDP. Decrease in fluorescence was measured at intervals of 15 or 20 seconds. Datapoints shown represent the mean of 20 subsequent measurements. c; Rap1A was cotransfected in Cos-7 cells with the indicated full length GEF constructs. Cells were stimulated with forskolin (20 µM) and IBMX (1 mM) for 10 min and Rap1 activation was measured using GST-RalGDS-RBD as an activation-specific probe. d; HA-Rap2A was cotransfected in Cos-7 cells with the indicated full-length GEF constructs. Cells were labeled with 32P-orthophosphate, Rap2A was immunoprecipitated using 12CA5 monoclonal antibodies, nucleotides were eluted and separated by TLC and GTP/GDP ratios were measured using a phospho-imager.

[0059] Figure 6. Different cAMP-binding sites in Epac1 and Epac2. a; alignment of different cAMP-binding pockets and schematic presentation and purification of GST-fusion constructs containing the cAMP-binding domains of Epac1 (B) and Epac2 (A and B) and the regulatory domain of Epac2 (RD) (coomassie-stained gel). b,c; The affinities of the isolated cAMP-binding sites in Epac1 and Epac2 were determined by ITC (see experimental procedures). The upper part of the graphs show the time dependent heating power detected after each injection of cAMP. In the lower part, the integrated heating power is normalized to the concentration of injected cAMP and plotted against the molar ratio of the nucleotide and the protein. The conditions used for the different constructs were: 59 µM cAMP-binding domain of Epac1 titrated with 0.72mM cAMP; 200 µM cAMP-binding site A of Epac2 with 3.7 mM cAMP; 34 µM cAMP-binding site B of Epac2 with 0.36 mM cAMP; 68 µM of the complete regulatory domain of Epac2 with 0.68 mM cAMP and in the mixture, 200 µM each of site-A and site-B of Epac2 with 3.7 mM cAMP. d; Kd's (µM) that are calculated from the ITC measurements in b and c are summarized in a table.

[0060] Figure 7. Regulation of Epac catalytic activity by isolated B-sites.

a; Schematic representation and purification (coomassie-stained gel) of the GST-fusion constructs used in b,c and d.

b; Epac1-DDEP or Epac2-DDEP was incubated at approximately 50 nM with purified Rap1A loaded with mantGDP (100 nM). cAMP (10 µM) was added at the indicated timepoints. c; Isolated catalytic domains of Epac1 or Epac2 (50nM) were incubated with their respective regulatory domains (Epac1-RD at 150 nM, Epac2-RD at 3 µM) and mantGDP loaded Rap1A (100nM). cAMP (100 µM) was added at the indicated time points. In the left panel measurements of mantGDP-loaded Rap1 alone (open circles), or in the presence of only the catalytic domain of Epac1 (open squares) are shown for comparison. d; The catalytic domain of Epac2 (50 nM) is incubated with the isolated cAMP-binding domains of Epac 2 (A-site at 200 µM and B-site at 3µM) and mantGDP loaded Rap1A (100 nM). 100 µM cAMP was added to the incubation with the B-site at the indicated timepoint.

[0061] Figure 8. The regulatory domain of Epac1 can block the catalytic domain of closely related RapGEFs. a; Purification of GST-fusions of the catalytic and regulatory domains used in b. C3G was cleaved from the GST-tag and purified by gelfiltration. b; Intrinsic exchange of mantGDP-loaded Rap1A (100 nM), or Rap2A in the case of PDZ-GEF1, (open circles), activation by the indicated catalytic domains (50 nM) (open squares)

and inhibition or lack of inhibition by the presence of Epac1-RD at 150 nM, or 300 nM in the case of C3G and CalDAG-GEFI, or PKA-RIa at 500 nM (black circles).

[0062] Figure 9. CalDAG-GEF contains an autoinhibitory domain regulated by calcium. Equal amounts of CalDAG-GEF and mutant CalDAG-GEF lacking the DAG and Ca binding domains were incubayed with mantGDP-loaded Rap1 and incubated in the presence or absence of 100 μM calcium.

[0063] Figure 10. PDZ-GEF contains an auto-inhibitory domain regulated by a factor present in Jurkat lysate and induced by serum.

[0064] Figure 11. Currently established guanine nucleotide exchange proteins for Rap

References

[0065]

Andrew, D., A. Shock, E. Ball, S. Ortlepp, J. Bell, and M. Robinson. 1993. *Eur. J. Immunol. 9*: 2217-2222.

Bos, J. L. 1997. *Biochim. Biophys. Acta*. 1333: 19-31.

Bos, J.L. 1998. EMBO J. 17:6776-6782

Cabanas, C., and N. Hogg. 1993. *Proc. Natl. Acad. Sci., U.* S. *A.* 90: 5838-5842.

De Rooij, J., and J. L. Bos. 1997. *Oncogene*. 14: 623-625.

De Rooij, J.,N.M. Boenink, M. van Triest5,R.H. Cool,A. Wittinghofer, and J.L. Bos. 1999. *J Boil Chem*.274:38125-38130.

De Vires-Smits, A.M., L. van der Voorn, J. Downward, and J.L. Bos. 1995. *Methods Enzymol.* 255: 156-161.

Franke, B.,J.W. Akkerman, and J.L. Bos. 1997. *EMBO J.* 16:252-259

Dransfield, I., and N. Hogg. 1989. *EMBO J.* 8: 3759-3765.

Gotoh, T., S. Hattori, S. Nakamura, H. Kitayama, M. Noda, Y. Takai, K. Kaibuchi, H. Matsui, o. Hatase, H. Takahashi, and et al. 1995. *Mol Cell Biol.* 15: 6746-6753.

Hughes, P.E. et al., 1997. *Cell.* 88:521-530

Hughes, P.E. and Pfaff, M., 1998. *Trends Cell Biol.* 8:359-364 Kawasaki, H., G.M. Springett, S. Toki, J. J. Canales, P. Harlan, J.P. Blumenstiel, E.J. Chen, I.A. Bany, N. Mochizuki, A. Ashbacher, M. Matsuda, D.E. Housman, and A.M. Graybiel. 1998._*Proc Natl Acad Sci U S A.* 95:13278-13283.

Kolanus, W., and Seed, B. 1997. *Curr. Opin. Cell Biol.* 9:725-731.

Kitayama, H., Y. Sugimoto, T. Matsuzaki, Y. Ikawa, and M. Noda. 1989. *Cell.* 56:77-87.

Kurachi, H., Y. Wada, N. Tsukamoto, M. Maeda, H. Kubota, M. Hattori, K. Iwai, and N. Minato. 1997. *J. Boil. Chem.* 272:28081-28088.

Liao, Y., K. Kariya, C.D. Hu, M. Shibatohge, M. Goshima, T. Okada, Y. Watari, X. Gao, T.G. Jin, Y. Yamawaki-Kataoka, and T. Kataoka. 1999. *J. Boil. Chem.* 274:37815-37820.

Liu, Z.-J., Y. Tanaka, H. Fujimoto, S. Mine, A. Morinobu, H. Yagita, K. Okumura, I. Oishi, J. Udagawa, H. Yamamura, and Y. Minami. 1999. *J. Immunol.* 163:4901-4908.

Medema, R.H., R. Wubbolts, and J.L. Bos. 1991. *Mol. Cell. Boil.* 11:5963-5967.

Newton, J.P., C.D. Buckley, E.Y. Jones, and D.L. Simmons. 1997. *J. Boil. Chem.* 272:20555-20563.

Ohtsuka, T., Y. Hata, N. Ide, T. Yasuda, E. Inoue, T. Inoue, A. Mizoguchi, and Y. Takai. 1999. *Biochem Biophys res Commun.* 265:38-44.

O'Rourke, A.M., H. Shao, and J. Kaye. 1998. *J. Immunol.* 161:5800-5803.

Reedquist, K.A., and J.L. Bos. 1998. *J. Boil. Chem.* 273:4944-4949.

Rubinfield, B., S. Munemitsu, R. Clark, L. Controy, K. Watt, W.J. Crosier, F. McCormick, and P. Polakis. 1991. *Cell.* 65-1033-1042.

Ruoslathi, E. 1999. *Adv. Canc. Res.* 65:1-29

Shibayama, H., N. Anzai, S.E. Braun, S. Fukuda, C. Mantal, and H.E. Broxmeyer. 1999. *Blood.* 93: 1540-1548.

Stewart, M., and N. Hogg. 1996. Regulation of leukocyte intergrin function: affinity vs advidity. *J. Cell. Biochem.* 61:554-561.

Su, Y., W.R. Dostmann, F.W. Herberg, K. Durick, N. H. Xuong, L. Ten Eyck, S.S. Taylor, and K.I. Varughese. 1995. *Science.* 269:807-813

Tanaka, S., T. Morishita, Y. Hashimoto, S. Hattori, S. Nakamura, T. Takenawa, K. Matuoka, M. Shibuya, T. Kurata, K. Nagashima, and M. Matsuda. 1994. *Proc. Natl. Acad. Sci. USA.* 91:3443-3447.

Tsukamoto, N., M. Hattori, H. Yang, J.L. Bos, and N. Minato. 1999. *J. Boil. Chem.* 274:18463-18469.

Van den Berghe,N., R.H. Cool, G. Horn, and A. Wittinghofer. 1997. Oncogene. 15:845-850.

Van de Wiel-van Kemenade, E., Y van Kooyk, A. J. de Boer, R. J. Huijbens, P. Weder, W. van de Kasteele, C. J. Melief, and C. G. Figdor. 1992. *J. Cell Biol.* 17: 461-470.

Yan, H. C., J. M. Pilewski, Q. Zhang, H. M. DeLisser, L. Romer, and S. M. Albelda. 1995. *Cell. Adhes. Commun.* 3: 45-66.

Zhang, A., K. Vuori, H. G. Wang, J. C. Reed, and E. Ruoslahti. 1996. *Cell.* 85:61-69.

Zwartkruis, F. J. T., R. M. F. Wolthuis, N. M. J. M. Nabben, B. Franke, and J. L. Bos. 1998. *EMBO J.* 17: 5905-5912.

Claims

1. A method for modulating cell adhesion comprising modulating a protein belonging to the Rap family of small GTPases.

2. A method for modulating cell adhesion comprising modulating a guanine nucleotide exchange factor for a protein belonging to the Rap family of small GTPases.

3. A method according to claim 1 or 2 wherein modulating said cell adhesion comprises modulating integrin-mediated events in said cell adhesion.

4. A method according to claim 3 wherein modulating said cell adhesion comprises modulating the auto-inhibitory domain of a guanine nucleotide exchange factor for a protein belonging to the Rap family of small GTPases.

5. Use of a modulator of a protein belonging to the Rap family of small GTPases for the preparation of a medicament for adhesion or anti-adhesion therapy.

6. Use of a modulator of a guanine nucleotide exchange factor for a protein belonging to the Rap family of small GTPases for the preparation of a medicament for adhesion or anti-adhesion therapy.

7. Use according to claim 5 or 6 wherein adhesion or anti-adhesion therapy comprises modulating integrin-mediated events.

8. Use according to claim 7 wherein said modulator comprises a modulator of the auto-inhibitory domain of a guanine nucleotide exchange factor of a protein belonging to the Rap family of small GTPases.

9. A method for identifying a modulator of a protein belonging to the Rap family of small GTPases or of modulator of a guanine nucleotide exchange factor for a protein belonging to the Rap family of small GTPases comprising

   a) providing for binding of a nucleotide or nucleotide analogue to a protein belonging to the Rap family of small GTPases,
   b) incubating said protein in the presence of a guanine nucleotide exchange factor of a protein belonging to the Rap family of small GTPases and
   c) incubating said protein in the presence of a candidate modulator compound and
   d) measuring release of said nucleotide or nucleotide analogue from said protein belonging to the Rap family of small GTPases.

10. A method according to claim 9 wherein activation of said release is measured.

11. A method according to claim 9 wherein inhibition of said release is measured.

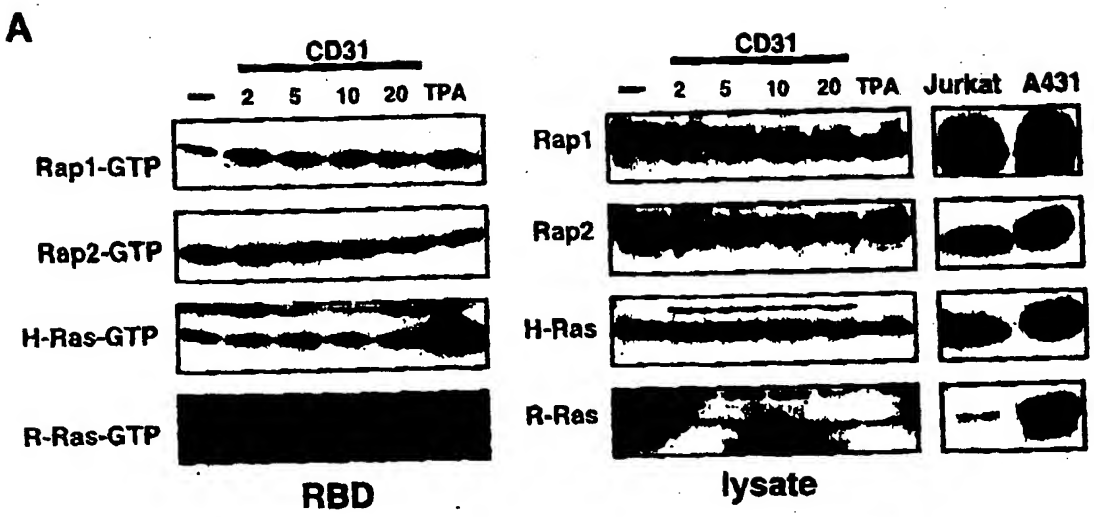
12. A modulator obtainable by a method according to anyone of claims 9 to 11.

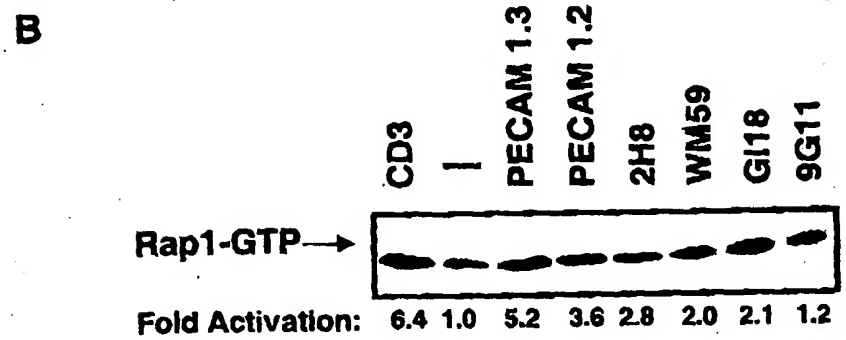
13. Use of a modulator according to claim 12 in a method for modulating cell adhesion.

14. Use of a modulator according to claim 12 for the preparation of a medicament.

15. Use according to claim 14 for the preparation of a medicament for adhesion or anti-adhesion therapy.

16. A medicament comprising a modulator according to claim 12.


A
CD31
— 2 5 10 20 TPA
Rap1-GTP
Rap2-GTP
H-Ras-GTP
R-Ras-GTP
RBD
CD31
— 2 5 10 20 TPA Jurkat A431
Rap1
Rap2
H-Ras
R-Ras
lysate


B
CD3
—
PECAM 1.3
PECAM 1.2
2H8
WM59
GI18
9G11
Rap1-GTP
Fold Activation: 6.4 1.0 5.2 3.6 2.8 2.0 2.1 1.2

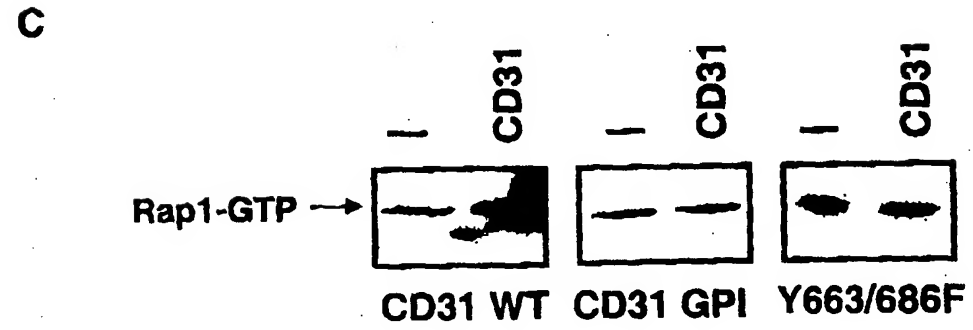

C
— CD31 — CD31 — CD31
Rap1-GTP
CD31 WT
CD31 GPI
Y663/686F

Figure 1:

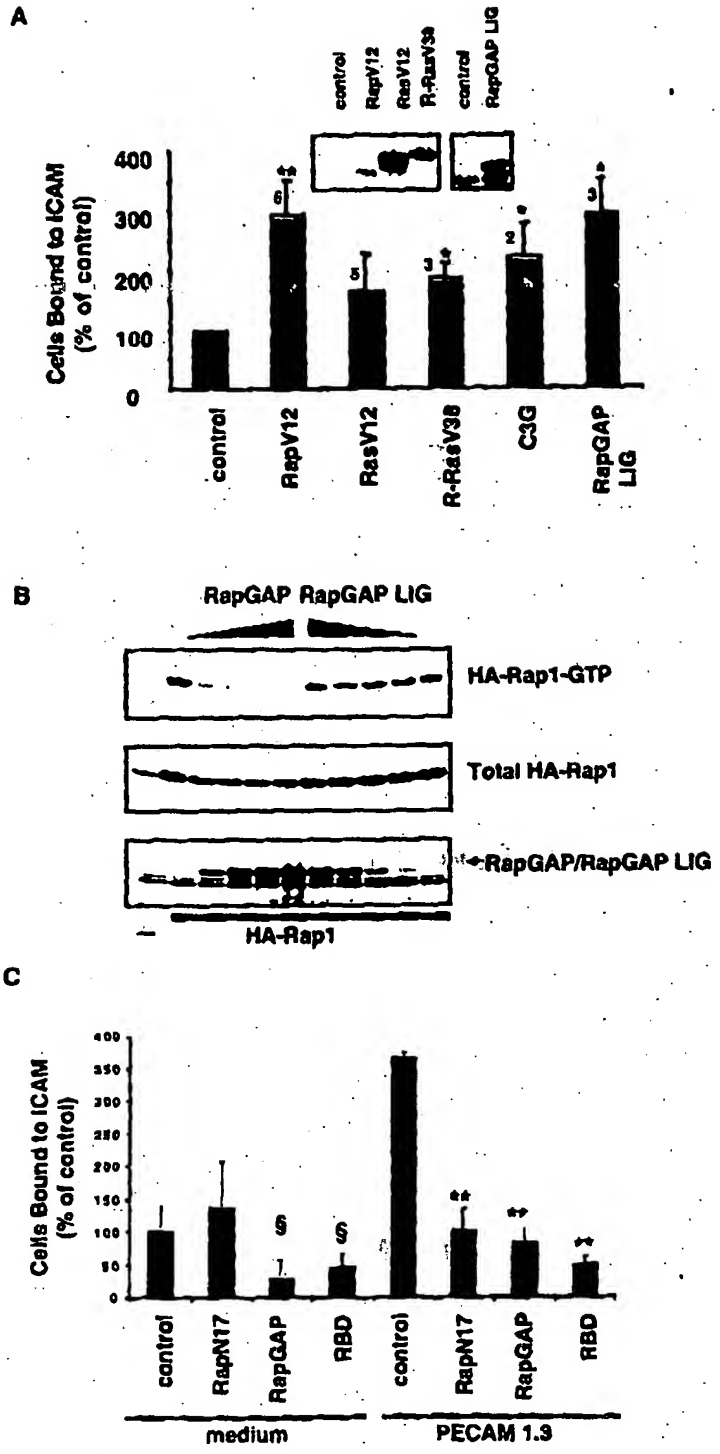

A
control
RapV12
RasV12
R-RasV38
control
RapGAP LIG
Cells Bound to ICAM (% of control)
400
300
200
100
0
control
RapV12
RasV12
R-RasV38
C3G
RapGAP LIG
B
RapGAP
RapGAP LIG
HA-Rap1-GTP
Total HA-Rap1
RapGAP/RapGAP LIG
HA-Rap1
C
Cells Bound to ICAM (% of control)
control
RapN17
RapGAP
RBD
control
RapN17
RapGAP
RBD
medium
PECAM 1.3

Figure 2:

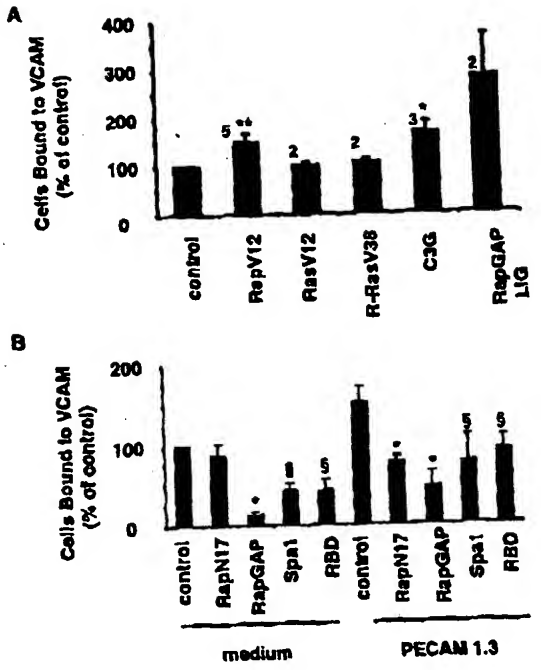

A
Cells Bound to VCAM (% of control)
400
300
200
100
0
control
RapV12
RasV12
R-RasV38
C3G
RapGAP 1/G
B
Cells Bound to VCAM (% of control)
200
100
0
control
RapN17
RapGAP
Spa1
RBD
control
RapN17
RapGAP
Spa1
RBD
medium
PECAM 1.3

Figure 3AB:

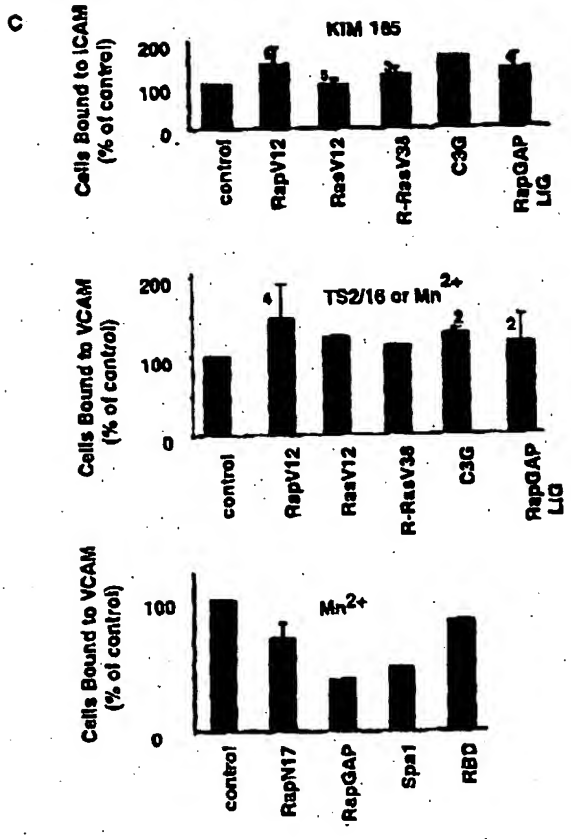

C
KIM 185
Cells Bound to ICAM (% of control)
200
100
0
control
RapV12
RasV12
R-RasV38
C3G
RapGAP LIG
TS2/16 or Mn2+
Cells Bound to VCAM (% of control)
Mn2+
RapN17
RapGAP
Spa1
RBD

Figure 3C:

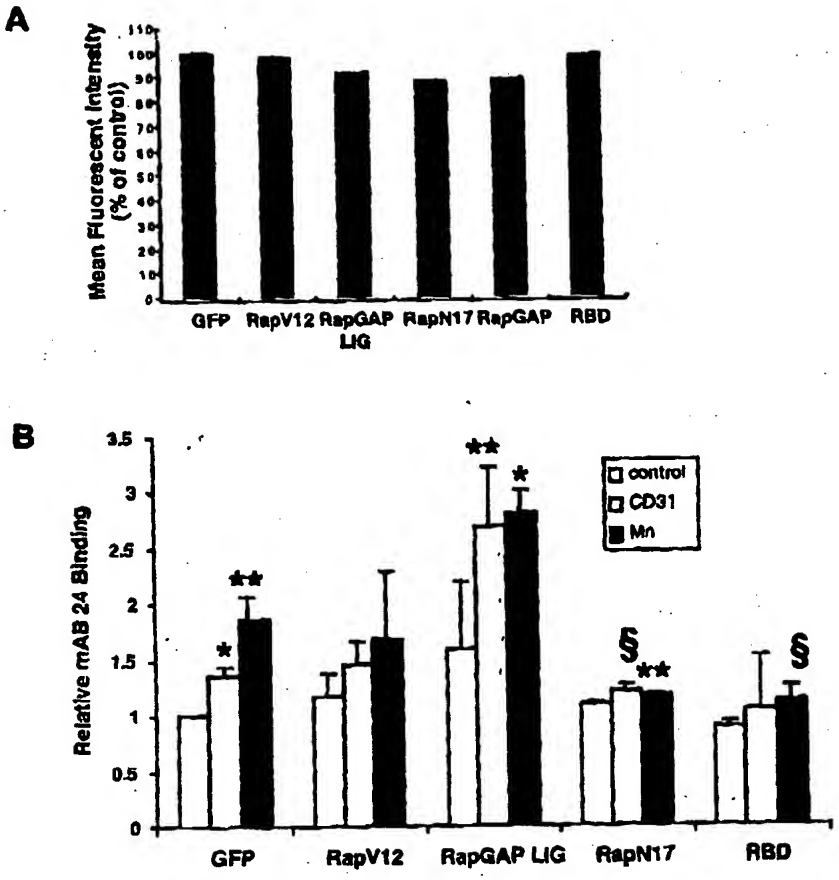
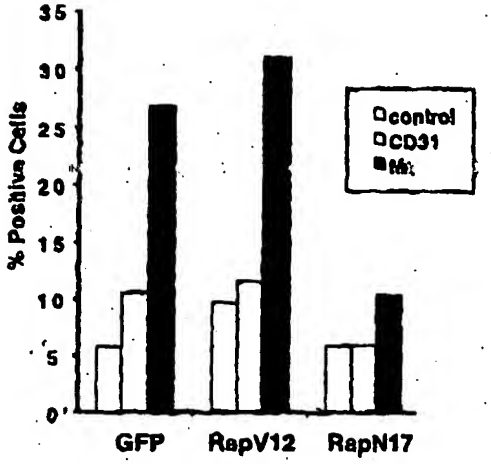

A
Mean Fluorescent Intensity (% of control)
GFP
RapV12
RapGAP LIG
RapN17
RapGAP
RBD
B
Relative mAB 24 Binding
control
CD31
Mn
GFP
RapV12
RapGAP LIG
RapN17
RBD

Figure 4AB

C



D

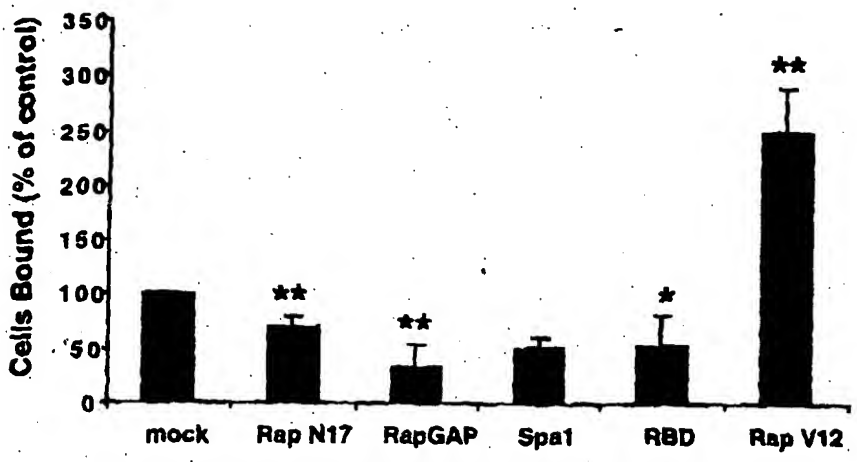
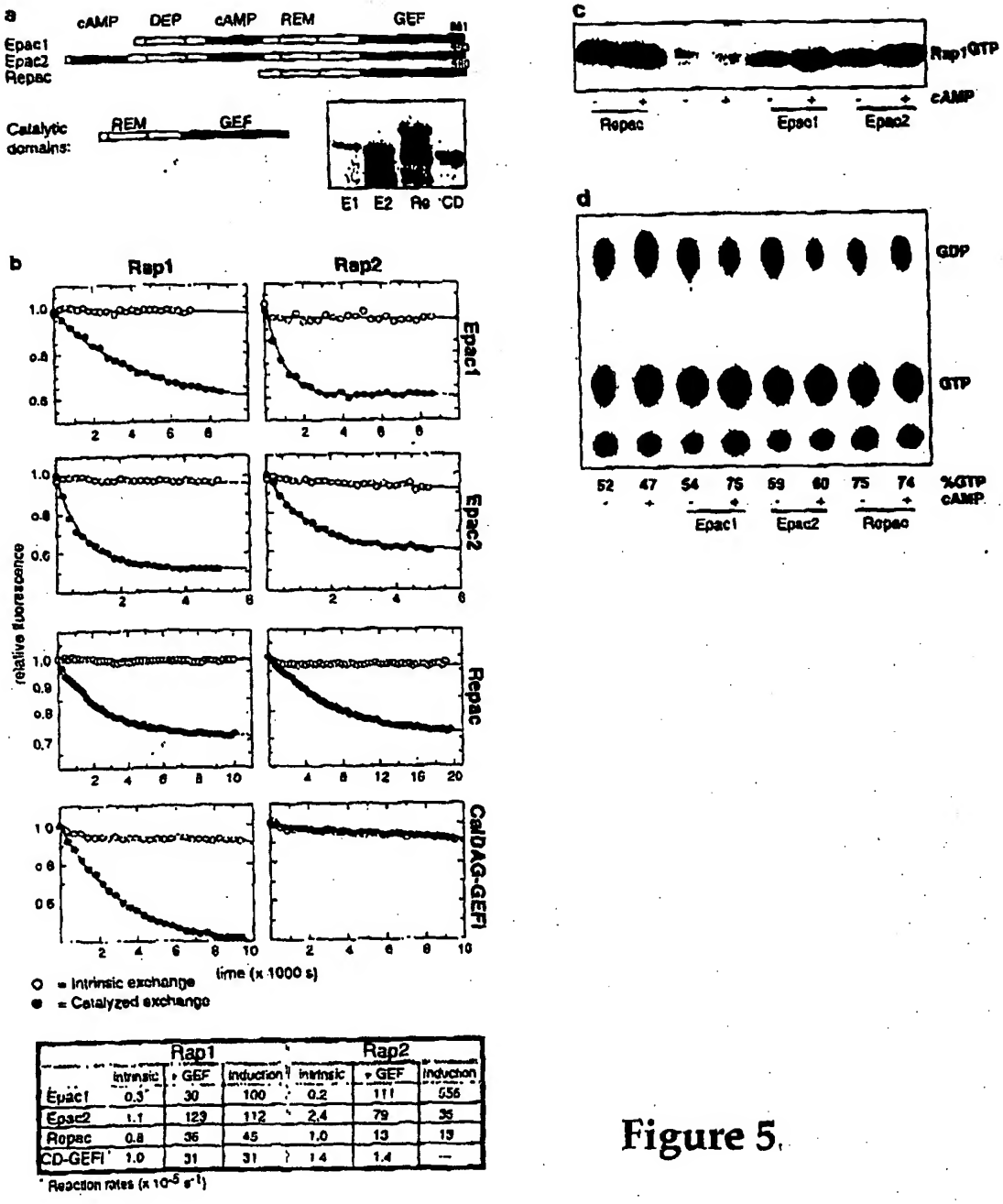


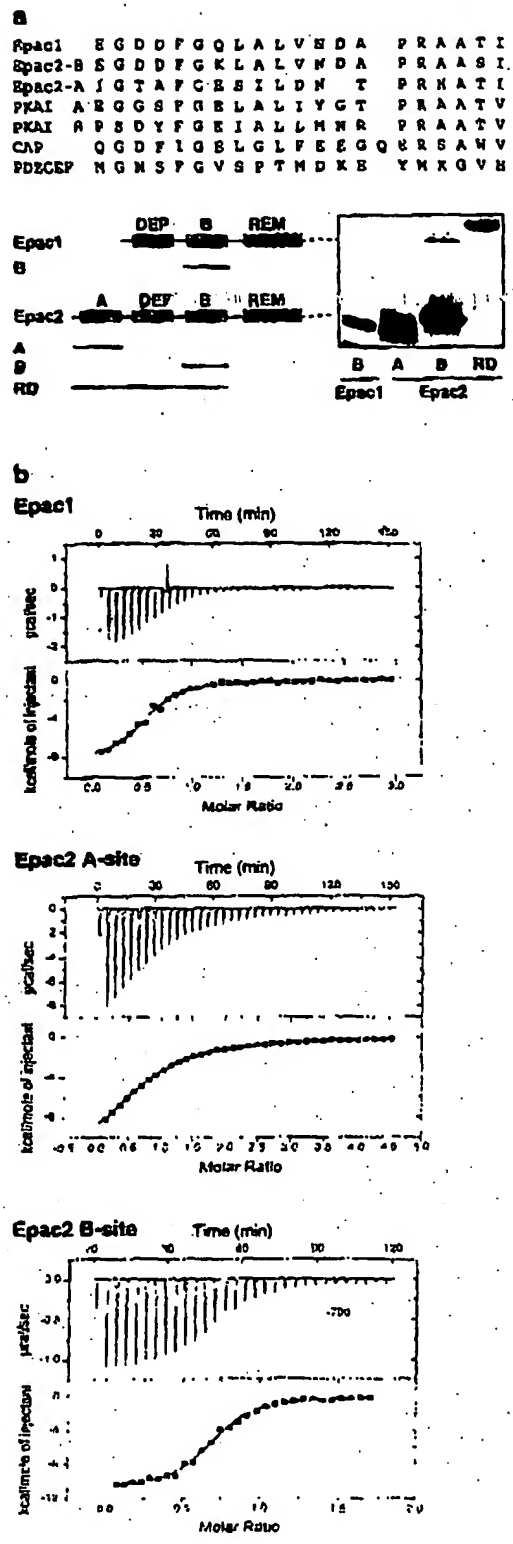
Figure 4CD:



	Rap1			Rap2		
	Intrinsic	+ GEF	Induction	Intrinsic	+ GEF	Induction
Epac1	0.3	30	100	0.2	111	556
Epac2	1.1	123	112	2.4	79	35
Repac	0.8	36	45	1.0	13	13
CD-GEFI	1.0	31	31	1.4	1.4	—

* Reaction rates (x 10^{-5} s^{-1})

Figure 5.



d

	Separate domains		
	Epac1	Epac2-A	Epac2-B
K_d*	4.0	87	1.2

	Epac2-RD		Mixed domains	
	1st Kd	2nd Kd	A-site	B-site
K_d*	77	0.5	76	0.6

* in M

Figure 6:

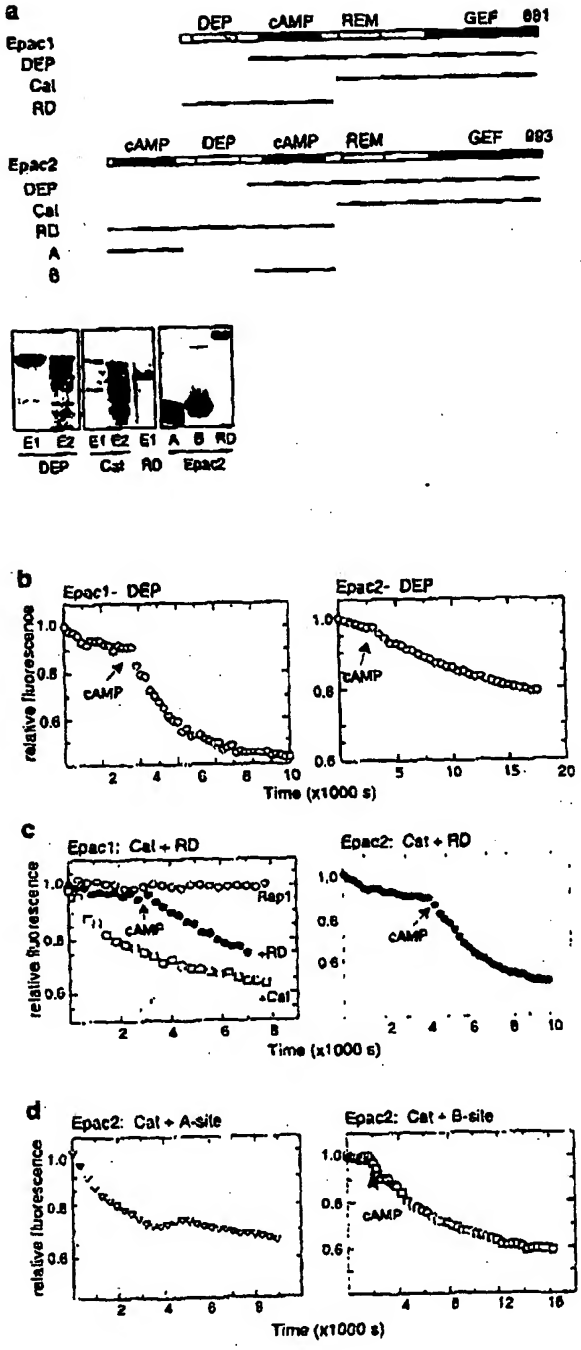

a
Epac1
DEP
Cat
RD
DEP
cAMP
REM
GEF
881
Epac2
DEP
Cat
RD
A
B
cAMP
DEP
cAMP
REM
GEF
993
E1
E2
E1
E2
E1
A
B
RD
DEP
Cat
RD
Epac2
b
Epac1- DEP
Epac2- DEP
relative fluorescence
cAMP
Time (x1000 s)
c
Epac1: Cat + RD
Epac2: Cat + RD
Rap1
+RD
+Cat
cAMP
Time (x1000 s)
d
Epac2: Cat + A-site
Epac2: Cat + B-site
relative fluorescence
cAMP
Time (x1000 s)

Figure 7:

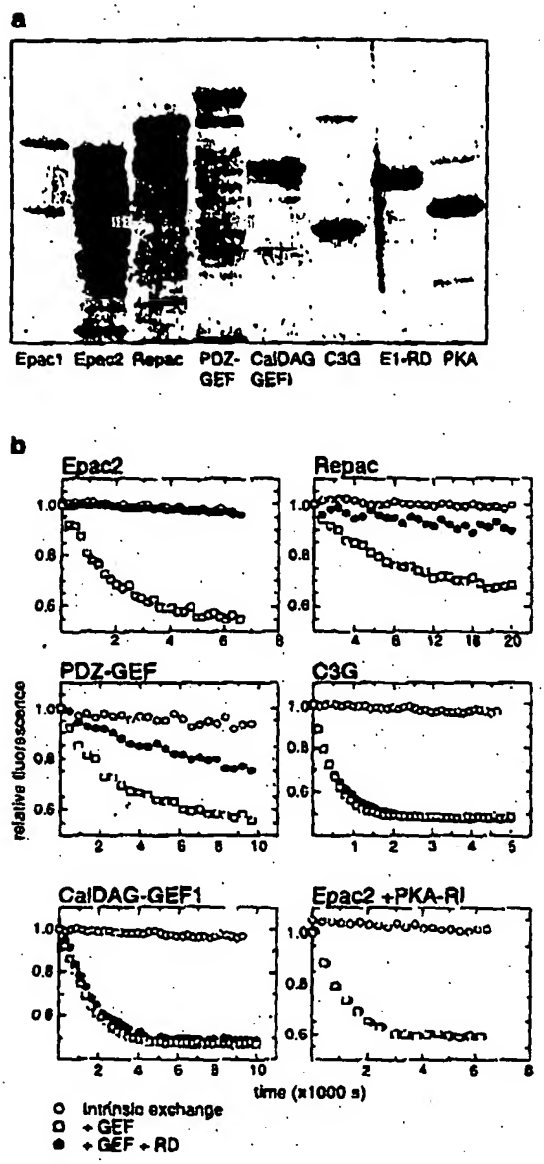
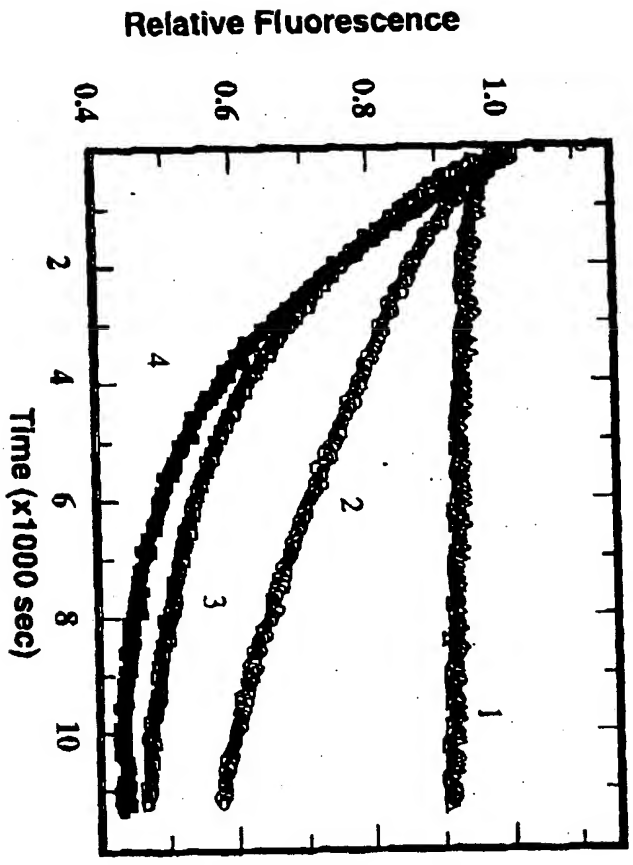

a
Epac1 Epac2 Repac PDZ-GEF CalDAG-GEF1 C3G E1-RD PKA
b
Epac2
Repac
PDZ-GEF
C3G
CalDAG-GEF1
Epac2 +PKA-RI
relative fluorescence
time (x1000 s)
Intrinsic exchange
+ GEF
+ GEF + RD

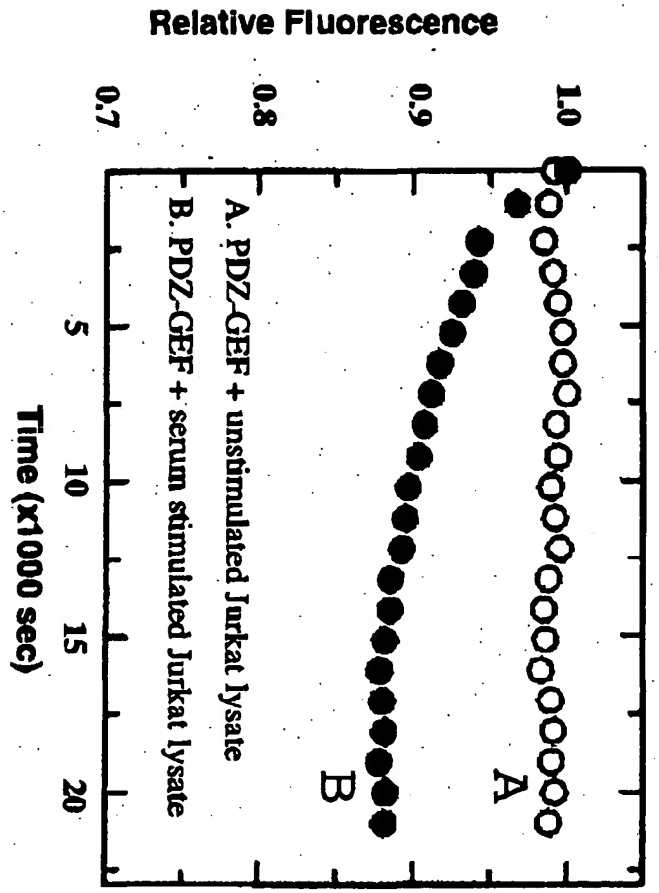
Figure 8:

Figure 9:



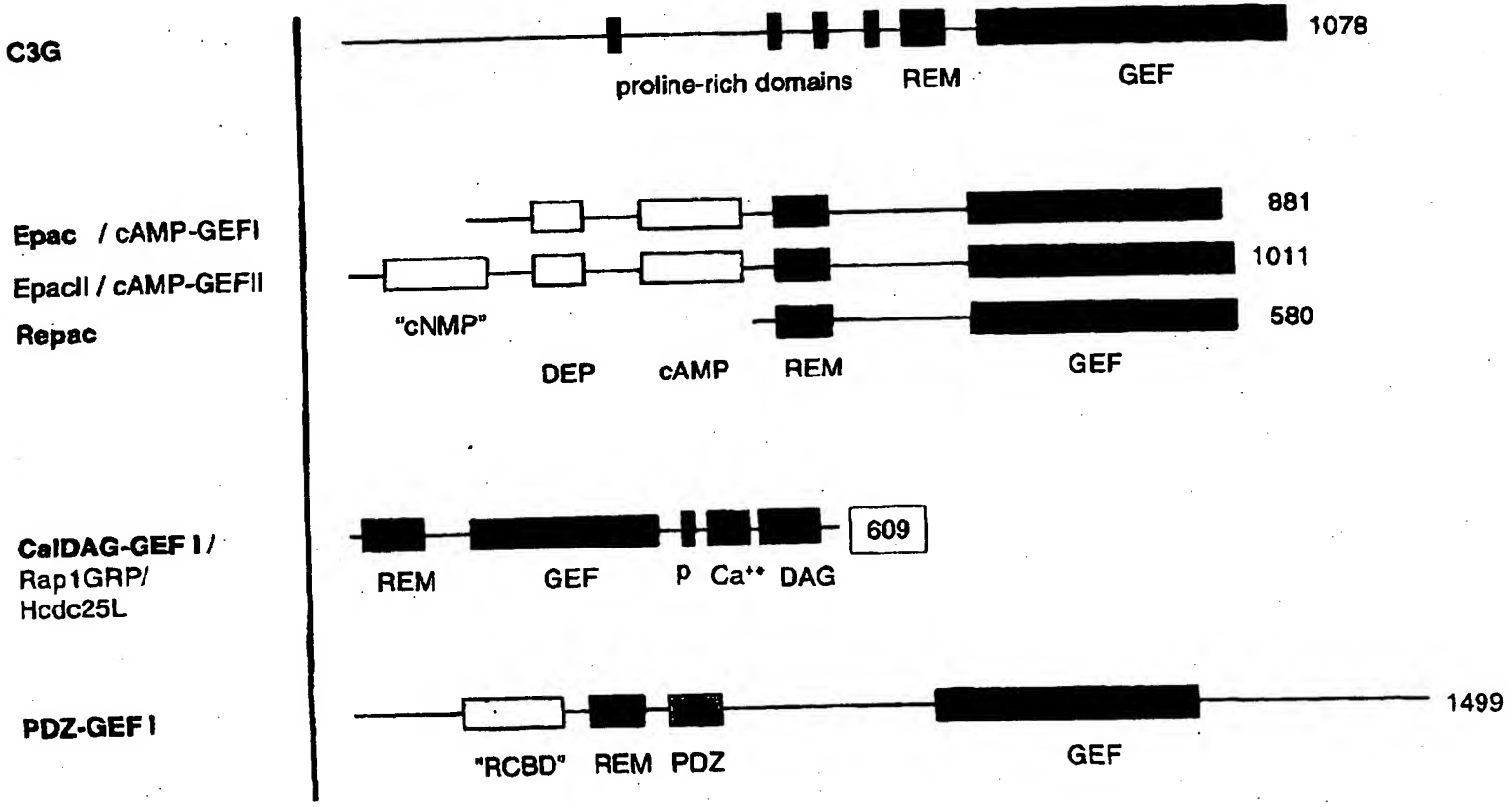
1. Intrinsic
2. Full-length CalDAG-GEF
3. Full-length CalDAG-GEF + Ca2+
4. CalDAG-CAT

Figure 10:


Relative Fluorescence
1.0
0.9
0.8
0.7
A
B
A. PDZ-GEF + unstimulated Jurkat lysate
B. PDZ-GEF + serum stimulated Jurkat lysate
5
10
15
20
Time (x1000 sec)

Rap1-specific GEFs

Figure 11:



European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 00 20 0567

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
D,X	FRANKE B ET AL: "Sequential regulation of the small GTPase Rap1 in human platelets." MOLECULAR AND CELLULAR BIOLOGY, (2000 FEB) 20 (3) 779-85. , XP002144700 * page 779, left-hand column, line 1 - right-hand column, line 2 *	1-16	C07K14/47 C12N9/16 A61K38/18 A61K38/46 C12Q1/34 A61P43/00
D,X	TSUKAMOTO N ET AL: "Rap1 GTPase-activating protein SPA-1 negatively regulates cell adhesion." JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 JUN 25) 274 (26) 18463-9. , XP002144701 * the whole document *	1-16	
D,A	DE ROOIJ J ET AL: "PDZ-GEF1, a guanine nucleotide exchange factor specific for Rap1 and Rap2." JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 DEC 31) 274 (53) 38125-30. , XP002144702 * abstract *	4	

-/--

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

C07K
C12N
A61K
C12Q
A61P

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

Although claims 1-4 (as far as an in vivo method is concerned) are directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

Place of search	Date of completion of the search	Examiner
THE HAGUE	10 August 2000	Le Flao, K

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 00 20 0567

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
D,A	KAWASAKI H ET AL: "A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia 'published erratum appears in Proc Natl Acad Sci U S A 1999 Jan 5;96(1):318!." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 OCT 27) 95 (22) 13278-83. , XP000882748 * abstract *	1-16	
T	DOWNWARD J: "Signal transduction. New exchange, new target 'news;comment!." NATURE, (1998 DEC 3) 396 (6710) 416-7. , XP002144703 * the whole document *	1-16	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	DE ROOIJ J ET AL: "Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP 'see comments!." NATURE, (1998 DEC 3) 396 (6710) 474-7. , XP002144705 * abstract *	1-16	
P,X	REEDQUIST K A ET AL: "The small GTPase, Rap1, mediates CD31-induced integrin adhesion." JOURNAL OF CELL BIOLOGY, (2000 MAR 20) 148 (6) 1151-8. , XP002144704 * abstract *	1-16	

EPO FORM 1503 03.82 (P04C10)

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)